

CALCIUM-DEPENDENT PROTEIN KINASE:
ACTIVATION MECHANISM AND INTERACTING PROTEINS

By

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To my father and late mother

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The activity of calmodulin-like domain protein kinase (CDPK) is regulated by the direct binding of Ca^{2+} . Unmodified soybean CDPK α and a chimeric enzyme in which the calmodulin-like domain (CLD) was replaced by VU-1 calmodulin had similar values of V_{maxapp} (3.19, 3.46 and 3.60, 3.93 $\mu\text{mol}/\text{min}/\text{mg}$, respectively), and each was activated 30- to 70-fold by Ca^{2+} . To ask if activation results from the binding of the CLD to the autoinhibitory (junction) domain of CDPK α in a manner analogous to the activation of calmodulin-dependent enzymes by calmodulin, recombinant CLD and truncation mutants of CDPK α were expressed in bacteria and highly purified. In blot overlays, biotinylated CLD bound to mutants containing residues 312-328 of the junction domain. In an electrophoretic mobility shift assay CLD bound synthetic peptides containing residues 318-332 in a calcium-dependent

manner, providing direct evidence for binding of CLD to a site in the junction domain. Mutants of CDPK α from which all or part of the CLD had been deleted were constitutively inactive. Addition of 20 μ M CLD to these mutants in the presence, but not the absence, of calcium stimulated their activities, but to various degrees. His₆-CDPK α (1-328), which contained none of the CLD, was activated only 5-fold, but the activity of His₆-CDPK α (1-398), which retained nearly half of the CLD in its sequence, was stimulated 64-fold. The latter activity approached that of unmodified CDPK α and was half maximal at a CLD concentration of 7 μ M. Our results suggest that binding of CLD to the junction domain contributes to, but is not sufficient for activation. Although calmodulin supported full activity of the chimeric enzyme, its addition to His₆-CDPK α (1-398) resulted in activity that was only 6% of that of the unmodified enzyme and which was half maximal at 20 μ M *Arabidopsis* calmodulin. These results support the conclusion that simple binding of the calmodulin-like domain to the junction domain is not sufficient for activation.

To identify the interacting proteins of CDPKs, interaction cloning was employed. GST-CDPK α , β , and γ were autophosphorylated and used as probes. Twelve interacting clones were isolated and characterized. Eight out of twelve were identified from a database search. Four of them were phosphorylated by CDPKs. One of the interacting proteins, soybean serine acetyltransferase (sSAT), which is involved in L-cysteine biosynthesis, showed that the phosphorylation affected the allosteric regulation. Phospho-MBP-sSAT was insensitive to feedback inhibition by L-cysteine. The stoichiometry was 0.96 mol of phosphate/mol of MBP-sSAT. These data suggest that the plant may have yet another means to regulate L-cysteine biosynthesis.

CHAPTER 1 LITERATURE REVIEW

Introduction

Calcium has been well recognized as an intracellular second messenger in plants. Transient changes in intracellular free calcium concentrations have been connected to many signal transduction pathways in response to a number of physiological stimuli, including light, touch, wind, pathogenic elicitors, cold-shock, gravity, and hormones such as abscisic acid, auxin, cytokinins, ethylene, and gibberellic acid (Bush, 1993, 1995, Gilroy et al., 1993, Poovaiah and Reddy, 1993, Raz and Fluhr, 1992, Trewavas and Knight, 1994).

The resting level of the intracellular Ca^{2+} concentration is maintained at the very low level (30 - 100 nM), while during stimulation the Ca^{2+} concentration in the cytoplasm increases as high as 10 μM , depending on stimuli (Trewavas and Knight, 1994). Although the precise mechanism that links these stimuli-induced changes in cytosolic Ca^{2+} concentration to the physiological responses has yet to be defined, understanding of calcium signal transduction pathways in plants has been furthered by the discovery of proteins that serve as the targets of calcium regulation. Among these are calmodulin and CDPKs (calmodulin-like domain protein kinase or calcium-dependent protein kinase) (Roberts and Harmon, 1992). These two proteins appear to be present ubiquitously in plants. Calmodulin does not have intrinsic enzymatic activity, but upon binding to calcium, it modulates activities of many other proteins. CDPK, on the other hand, has protein

kinase activity which is regulated by direct binding to calcium (Harmon et al., 1987, Putnam-Evans et al., 1990).

Structural Features and Sequences

The CDPK Family

Since a CDPK cDNA was first cloned from soybean (Harper et al., 1991), a large number of CDPK genes have been cloned from plants, including carrot (Suen and Choi, 1991), alfalfa (Monroy and Dhindsa, 1995), *Arabidopsis* (Harper et al., 1993, Hong et al., 1996, Hrabak et al., 1996, Urao et al., 1994), rice (Breviario et al., 1995, Kawasaki et al., 1993), maize (Estruch et al., 1994, Takezawa et al., 1996), and mungbean (Botella et al., 1996). Data from cDNA cloning, genomic southern analysis (Harper et al., 1991, Hong et al., 1996, Hrabak et al., 1996), and western analysis with monoclonal antibodies raised against native soybean CDPK (Harper et al., 1993) suggest that multiple isoforms of CDPKs are present in plants. There are also biochemical evidences indicating the presence of CDPKs in plants, such as zucchini (Verhey et al., 1993), groundnut (Dasgupta, 1994), rice (Abo-El-Saad and Wu, 1995), maize (Pestenacz and Erdei, 1996), sorghum (Pestenacz and Erdei, 1996), tobacco (Kunz et al., 1996, Ohto and Nakamura, 1995), potato (MacIntosh et al., 1996), *Vicia faba* (Li and Assmann, 1997), and winged bean (Saha and Singh, 1995). CDPKs are present in other organisms, as well. Zhao et al. (1993) cloned a CDPK gene from a protist, *Plasmodium falciparum*. In *Paramecium*, two CDPK isoenzymes have been purified to homogeneity (Gundersen and Nelson, 1987, Son et al., 1993). CDPK has been identified and localized in the green alga, *Chara* (Harmon and McCurdy, 1990, McCurdy and Harmon, 1992).

Structural Features

CDPKs are monomeric enzymes (Dasgupta, 1994, Harmon et al., 1987, Putnam-Evans et al., 1990, Son et al., 1993). CDPKs have a unique primary structure, which is well conserved among all known CDPKs (Fig. 1-1). They contain three basic functional domains; a kinase catalytic domain, a junction domain, and a calmodulin-like domain (listed in order from the amino terminus to the carboxyl terminus).

Kinase catalytic domain

The kinase catalytic domain of CDPK is most closely related in sequence to that of Ca^{2+} /calmodulin-dependent protein kinases (Roberts and Harmon, 1992). The catalytic domain is about 260 amino acid residues, corresponding to about 30 kD. It contains highly conserved 11 subdomains that would be expected to be present in protein kinases (Hanks et al., 1988, Hardie and Hanks, 1995, Harper et al., 1991). These subdomains have been strongly implicated to play essential roles in catalytic function as components of active site which directly participate in adenosine triphosphate (ATP) binding and phosphotransfer. They are also implicated in catalysis indirectly by contributing to the formation of the active site through constraints imposed on secondary structure (Hanks et al., 1988, Hardie and Hanks, 1995, Lei et al., 1994a).

In the past few years the crystal structures of several protein kinases have been determined (De Bondt et al., 1993, Goldberg et al., 1996, Hu et al., 1994b, Hubbard et al., 1994, Knighton et al., 1991, Longenecker et al., 1995, Owen et al., 1995, Zhang et al., 1994). They reveal that overall architectures of each protein kinase are very similar with each other. Furthermore, they provide good evidence that the functions of conserved subdomains are well

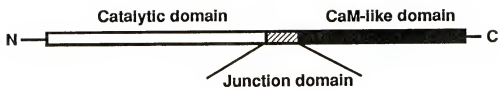


Figure 1-1. Structural organization of CDPK.

The domain structures of CDPK is shown schematically. The drawing is not in actual scale.

correlated with the structural organization of the catalytic core of protein kinase (Johnson et al., 1996, Lei et al., 1994a, Taylor et al., 1993).

Junction domain

The junction domain of CDPKs contains the autoinhibitory and calmodulin-binding domain (Harmon et al., 1994, Harper et al., 1994). An autoinhibitory domain refers in general to an inhibitory region that is part of primary structure of an enzyme (Hardie, 1988, Kemp et al., 1994, Kemp and Pearson, 1991, Kemp et al., 1991). An exception is protein kinase A (PKA) whose autoinhibitory domain resides on the regulatory subunit. Studies on several protein kinases such as protein kinase C, myosin light chain kinase (MLCK), calmodulin-dependent protein kinase (CaMPK) family, PKA, and phosphorylase kinase (PhK) have demonstrated that the autoinhibitory domain acts as a pseudosubstrate (Hardie, 1988, Kemp et al., 1994, Kemp and Pearson, 1991, Kemp et al., 1991). A pseudosubstrate refers to a structure that appears to be structurally similar to the specific substrate for the kinase but lacks a phosphorylatable residue. The simple pseudosubstrate hypothesis is that in an inactive state the pseudosubstrate folds back into the active site and creates a steric block that prevents access of the substrate.

Synthetic peptides corresponding to the pseudosubstrate region of several protein kinases act as competitive inhibitors of exogenous substrates (Kemp et al., 1991). Removal of an autoinhibitory domain by proteolysis results in constitutively active protein kinases (Hanson and Schulman, 1992, Pearson et al., 1988, Yamagata et al., 1991). The crystal structure of the kinase and regulatory domain of the large muscle protein, twitchin, has been solved in an autoinhibited form (Hu et al., 1994a, 1994b). A previous study on twitchin kinase revealed that a 60-residue sequence on the carboxyl-terminal tail of the catalytic core is responsible for the autoinhibition (Lei et al., 1994).

Similar molecular arrangements are found in CDPK, MLCK, and CaMPK, which are all autoinhibited by the carboxyl sequences of catalytic domain. In the crystal structure, the autoinhibitory domain is bound to and blocks the active site, which supports the pseudosubstrate hypothesis (Hu et al., 1994b). Recent determination of the crystal structure of CaMPK I, however, has added complexity to the hypothesis (Goldberg et al., 1996). Part of the regulatory domain of CaMPK I interferes with the binding site for peptide substrates, which is consistent with the hypothesis. The other part, however, appears to interact with the outside of the ATP-binding region instead of directly entering the ATP-binding site, leading to conformational changes that obstruct the nucleotide binding site.

Studies on soybean CDPK α and *Arabidopsis* CDPK, AK1, have demonstrated that CDPK is also regulated by the pseudosubstrate mechanism (Harmon et al., 1994, Harper et al., 1994). Synthetic peptides corresponding to residues 317-332 of the junction domain of soybean CDPK α competitively inhibit the enzyme activity with respect to a peptide substrate (Harmon et al., 1994). Removal of junction and calmodulin-like domains results in a constitutively active Ca²⁺-independent enzyme, but removal of only calmodulin-like domain ends up inactive enzyme (Harmon et al., 1994, Harper et al., 1994). Furthermore, these studies showed that the junction domain also contains a calmodulin-binding region, which resides on the carboxyl side of the autoinhibitory domain but partially overlaps it. A band-shift assay with synthetic peptides corresponding to the junction domain and soybean calmodulin suggested that a region corresponding to residues 318-332 of CDPK α is involved in binding to calmodulin. Combined together, these data suggest that the junction domain is an important regulatory element in CDPKs.

Calmodulin-like domain

The calmodulin-like domain resembles the calcium-binding protein, calmodulin, with 40% sequence identity to spinach calmodulin (Roberts and Harmon, 1992). It has four Ca^{2+} -binding motifs, EF hands (Roberts and Harmon, 1992). The EF hand was named arbitrarily after the E and F helices of parvalbumin (Kretsinger, 1980). This motif has been identified in numerous proteins and is considered as a prevailing motif for a Ca^{2+} -binding (Kawasaki and Kretsinger, 1994). The E and F helices are separated by a Ca^{2+} -binding loop to form a helix-loop-helix conformation. The Ca^{2+} -binding loop is generally composed of 12 amino acid residues which participate in Ca^{2+} coordination. In an EF hand the Ca^{2+} ion is coordinated by seven oxygen ligands. In general, five of these oxygens are provided by side chains, one by a backbone carbonyl, and one by a water molecule. A glycine residue in this loop is well conserved and important for the loop structure.

The EF hands in the calmodulin-like domain of CDPK have all the requirements for the Ca^{2+} -binding (Roberts and Harmon, 1992). Ca^{2+} -binding properties of CDPK were demonstrated by using $^{45}\text{CaCl}_2$ in a blot overlay assay (Dasgupta, 1994, Harmon et al., 1987, Harmon et al., 1996, Saha and Singh, 1995, Son et al., 1993). Furthermore, Zhao et al. (1994) showed that all four EF hands of *Plasmodium* CDPK bind Ca^{2+} . The presence of Ca^{2+} binding domains distinguishes CDPKs from other protein kinases that are regulated by Ca^{2+} and calmodulin, and confers the calcium sensitivity directly to the enzymes.

CDPKs with modified EF hands in the calmodulin-like domain have been reported. CDPKs from rice appear to contain only one functional EF hand based on sequence analysis (Breviario et al., 1995, Kawasaki et al., 1993). The other three EF hands contain amino acid substitutions in conserved

residues that are essential for Ca^{2+} binding. It will be interesting to see whether these CDPKs are capable of binding Ca^{2+} and activated by the Ca^{2+} . cDNAs encoding CRK (CDPK-related kinase) were cloned from carrot and maize (Furumoto et al., 1996, Lindzen and Choi, 1995). The primary structure of CRK is similar to typical CDPK, but all of four EF hands in CRK have diverged to varying extents from the canonical EF hand motif. Recombinant CRK is able to phosphorylate casein, but in the absence of Ca^{2+} (Furumoto et al., 1996), indicating this group of protein kinases differs from CDPK in activation kinetics. However, the function of the calmodulin-like domain in CRKs remains to be defined.

Biochemical Properties of CDPK

Calcium-dependent protein kinases are distinguished from Ca^{2+} /calmodulin-dependent protein kinases (CaMPK) (Hanson and Schulman, 1992, Schulman, 1988) and Ca^{2+} /phospholipid-dependent protein kinases (PKC) (Jaken, 1996) by the following properties: 1. dependence of activity on micromolar concentrations of free calcium; 2. direct binding of calcium and no requirement for calmodulin or phosphatidylserine for activity; and 3. ability to bind to hydrophobic matrices in a calcium-dependent manner (Roberts and Harmon, 1992).

Activation of CDPKs

Native soybean CDPK depends solely on micromolar free calcium and does not require calmodulin or phospholipid for activity (Harmon et al., 1987, Putnam-Evans et al., 1990). One hundred-fold stimulation of this enzyme is achieved by an addition of $>10 \mu\text{M}$ free calcium. The concentration of free calcium required for half-maximal activity ($K_{0.5}$) is $1.5 \mu\text{M}$ (Harmon et al.,

1987, Putnam-Evans et al., 1990). Calmodulin stimulates the enzyme activity by less than two fold at micromolar concentration. This stimulation seems to be non-specific because calmodulin activates enzymes at the nanomolar range and other proteins such as murine IgG and bovine serum albumin show the same level of stimulation (Harmon et al., 1987, Putnam-Evans et al., 1990). Similar properties have been observed for most other CDPKs (Dasgupta, 1994, MacIntosh et al., 1996, Roberts and Harmon, 1992, Saha and Singh, 1995, Son et al., 1993).

Although CDPKs from soybean, potato, and *Paramecium* are not affected by fatty acids or lipids (Gundersen and Nelson, 1987, MacIntosh et al., 1996, Putnam-Evans et al., 1990, Son et al., 1993), several CDPKs have been reported to be stimulated by fatty acids. CDPKs from wheat embryo and silver beet leaves show the stimulation by fatty acids such as oleic acid and linoleic acid (Klucis and Polya, 1987, Minichiello et al., 1989). Oat plasma-membrane CDPK is also stimulated by crude soybean lipid (Schaller et al., 1992). The stimulation observed in the presence of lipids is 20-fold higher than in the absence of lipids but in the presence of calcium. A recombinant CDPK encoded by AK-1 (*Arabidopsis* kinase-1) has been studied in detail for lipid regulation (Binder et al., 1994, Harper et al., 1993). The activity of this CDPK is stimulated synergistically by calcium and phospholipids. The effects of phospholipids are specific to phosphatidylinositol and lysophosphatidylcholine. It is not known, however, whether the lipid or fatty acid sensitivity of these CDPKs has any physiological significance.

Inhibition of CDPKs

Calmodulin antagonists, such as phenothiazines (chlorpromazine, trifluoperazine, fluphenazine), a naphthalene sulfonamide (W-7), and

clamidazolium, inhibit CDPKs (Dasgupta, 1994, MacIntosh et al., 1996, Roberts and Harmon, 1992, Saha and Singh, 1995, Son et al., 1993). It is known that the binding of Ca^{2+} to calmodulin induces a conformational change exposing hydrophobic groups (Roth et al., 1992). Hydrophobic and amphipathic antagonists bind to this hydrophobic region and prevent its binding and activation of the target enzymes. Considering the similarity of CDPK's regulatory domain to calmodulin, it is plausible to suggest that these compounds may bind to a hydrophobic region of this domain in a calcium-dependent manner and prevent activation of the enzyme.

Partially purified CDPKs from spinach leaves are inhibited by a variety of metabolic phosphate esters including dihydroxyacetone phosphate, glucose-6-phosphate, and fructose-1,6-bisphosphate but not by amino acids or by the Krebs cycle intermediates (Bachmann et al., 1995). It will be interesting to know whether these inhibitions are specific to these CDPKs since Lee and Harmon (unpublished results) observed no inhibitory effects of those compounds on soybean CDPKs at the concentration reported to be inhibitory.

Autophosphorylation

Several CDPKs have been observed to undergo autophosphorylation (Dasgupta, 1994, MacIntosh et al., 1996, Roberts and Harmon, 1992, Saha and Singh, 1995, Son et al., 1993, Verhey et al., 1993). The autophosphorylation of CDPKs is also dependent on calcium like the phosphorylation of their substrates. Bogré et al. (1988) observed that autophosphorylation of partially purified alfalfa CDPK increases enzyme activity four fold without changing the Ca^{2+} sensitivity. Autophosphorylation of native soybean CDPK stimulated by free calcium ($2\ \mu\text{M}$) occurs on serine and threonine residues, e.g. potato CDPKs (Verhey et al., 1993), but the effect of autophosphorylation is

not known (Harmon et al., 1987, Putnam-Evans et al., 1990). *Paramecium* CDPK also shows a similar autophosphorylation pattern with the half-maximal rate of autophosphorylation occurring at approximately 1-1.5 μM free calcium (Gundersen and Nelson, 1987, Son et al., 1993).

A CDPK from winged bean displays distinct autophosphorylation properties (Saha and Singh, 1995). Although the enzyme requires Ca^{2+} for substrate phosphorylation in vitro, it undergoes autophosphorylation independent of Ca^{2+} . Furthermore, the CaM antagonist, fluphenazine, which inhibits substrate phosphorylation, does not inhibit autophosphorylation. The stoichiometry reported is 1.04 mol of phosphate/mol of enzyme and it takes 90 min to reach an apparent saturation. The autophosphorylation is stimulated by histone H1 and MLC-peptide, 1.7 and 1.4 mol of phosphate/mol of enzyme, respectively, but not by syntide-2. The effect on autophosphorylation by substrates is due to the increase in V_{max} rather than decrease in K_m for ATP. The autophosphorylated enzyme is less active for substrate phosphorylation than the unautophosphorylated enzyme (Saha and Singh, 1995).

Physiological Roles of CDPK

Endogenous Substrates

Although a number of in vitro substrates for CDPKs have been reported (Roberts and Harmon, 1992), only a few potential endogenous substrates have been described. D. M. Roberts' group has found that nodulin 26, an integral membrane protein of the symbiosome membrane, is phosphorylated in vivo and in vitro by a CDPK which was partially purified from soybean root nodules (Weaver et al., 1991, Weaver and Roberts, 1992).

The phosphorylation occurs at only one site within the hydrophilic, cytoplasmic carboxyl terminal domain. In subsequent reports, they showed that nodulin-26 has an ion channel activity when purified nodulin 26 from soybean nodules was reconstituted into liposomes (Weaver et al., 1994), and phosphorylation of nodulin 26 by CDPK results in an increase in ion channel activity by modulating the voltage sensitivity of the channel (Lee et al., 1995).

Protein phosphorylation and multiple Ca^{2+} -dependent signaling pathways have been implicated in the control of stomatal guard cell aperture (Assmann, 1993). CDPK has been identified from guard cell protoplasts of *Vicia faba* and several soluble and membrane proteins of guard cells appeared to be phosphorylated by micromolar concentration of free Ca^{2+} (Li and Assmann, 1997). Pei et al. (1996) showed that a chloride channel in *Vicia faba* guard cell vacuoles is activated by recombinant *Arabidopsis* CDPK, AK1 (Harper et al., 1993) in patch-clamp experiments and suggested that the activation of the channel by phosphorylation contributes to the opening of guard cell by uptake of anion into vacuoles.

A seed-specific tonoplast intrinsic protein (TIP), which is a putative membrane channel, has been shown to be phosphorylated by tonoplast-bound protein kinase in a calcium-dependent manner (Johnson and Chrispeels, 1992). The phosphorylation occurs in vivo and in vitro at a serine residue on the amino terminus. The protein kinase responsible for phosphorylation is extractable from purified tonoplast by 0.5% triton X-100 or octyl-glucoside. The capacity to phosphorylate TIP changes during seed development but it is not addressed whether the changes in phosphorylation status of TIP occur due to changes in the availability of TIP or protein kinases. Neither a CDPK responsible for phosphorylation of TIP nor the role of phosphorylation on TIP has been demonstrated.

CDPKs have been identified to be required for modulation of nitrate reductase (NR), which is a cytosolic enzyme that reduces nitrate to nitrite (Bachmann et al., 1995, Bachmann et al., 1996b, Huber et al., 1996, McMichael et al., 1995). CDPK-dependent phosphorylation is followed by the binding of the inhibitor protein, 14-3-3, which results in inhibition of NR activity (Bachmann et al., 1996a, Glaab and Kaiser, 1995, Moorhead et al., 1996). The phosphorylation site has been assigned to a serine residue, which is located in a major regulatory domain of NR (Bachmann et al., 1996b, Douglas et al., 1995, Su et al., 1996). This serine is conserved in all of the known NR from dicots and monocots (Bachmann et al., 1996b). Two forms of CDPKs have been resolved and both are able to phosphorylate NR. These two CDPKs are similar in strict calcium-dependency for activity, but are distinct immunochemically (Bachmann et al., 1996b, McMichael et al., 1995). It is not known which form phosphorylates NR *in vivo*, or what controls them if both were involved.

Sucrose-phosphate synthase (SPS) is also regulated by phosphorylation/dephosphorylation (Huber et al., 1994). The phosphorylation occurs *in vivo* and *in vitro* exclusively on serine residues. It appears that one or two phosphorylation sites are responsible for the regulation of SPS activity. McMichael et al. (1995) have partially purified multiple protein kinases from spinach leaves. Upon anion-exchange and size-exclusion chromatography, these kinases are separated into three unique peaks that behave differently. Peaks containing 60 and 150 kD proteins were able to phosphorylate and inhibit SPS *in vitro*. The activity of the 60 kD protein kinase is dependent on micromolar concentration of Ca^{2+} whereas the 150 kD protein is not. It is premature to conclude 60 kD kinase is a CDPK

since the phosphorylation might have been occurred by calcium/calmodulin-dependent protein kinase.

Ca^{2+} -dependent protein phosphorylation has been linked to gametophytic self-incompatibility (Kunz et al., 1996, Rudd et al., 1996). Kunz et al. (1996) have found that a partially purified protein kinase from pollen tubes phosphorylates style S-RNases from incompatible species but not from compatible species. The protein kinase is inhibited by calmodulin antagonists and crossreacted with monoclonal antibodies raised against native soybean CDPK. They proposed CDPK is involved in self-incompatibility process by modulating S-RNase activity which has been confirmed to be sufficient for rejection of pollen of the same S-genotype (Kunz et al., 1996). By challenging pollen grown in vitro with biologically active recombinant S protein, Rudd et al. (1996) have demonstrated phosphorylation of a 26 kD pollen protein is increased in response to the self-incompatibility and the phosphorylation is dependent on Ca^{2+} . The phosphorylation is specific to challenge with incompatible S proteins, but not with compatible or incompatible heat-denatured S proteins.

Phosphatidylinositol 4-kinase-A49 (PIK-A49) is a multifunctional enzyme that has EF-1 α activity and acts as an activator of the plasma membrane PIK (Yang et al., 1993). PIK-A49 is phosphorylated in vitro by CDPK from carrot as well as soybean (Yang and Boss, 1995). The phosphorylation appeared to be essential for activation. Dephosphorylated PIK-A49 with alkaline phosphatase is unable to activate PIK but rephosphorylation of dephosphorylated PIK-A49 restores its full activity. The phosphorylation, however, does not affect the elongation factor activity of PIK-A49.

Schaller and Sussman (1988) demonstrated that calcium stimulates phosphorylation of plasma-membrane H^+ -ATPase from oat roots. Although the effect of phosphorylation on plant plasma-membrane H^+ -ATPase and whether CDPK phosphorylates it *in vivo* are yet to be demonstrated, the regulatory role of phosphorylation was suggested based on the observation that the stimulation of H^+ -ATPase occurs very rapidly and the phosphorylation of yeast plasma-membrane H^+ -ATPase stimulates activity (Schaller and Sussman, 1988).

Although the endogenous substrate of native soybean CDPK is not known, this enzyme has been implicated in the regulation of the cytoskeleton (Roberts and Harmon, 1992). Immunocytological studies have shown that this enzyme is colocalized with F-actin in plant root cells and pollen tubes (Putnam-Evans et al., 1989). A CDPK-like antigen is also colocalized with F-actin in *HeLa* cells (Harmon et al., 1989). Since the enzyme did not bind to the purified F-actin directly, it was proposed that the enzyme binds to an unidentified F-actin-binding protein (Putnam-Evans et al., 1989). In the green alga *Chara*, CDPK has been localized to the subcortical actin bundles, the surface of small organelles and other membrane components of the streaming endoplasm and proposed to be a putative mediator in the Ca^{2+} -induced inhibition of cytoplasmic streaming (Harmon and McCurdy, 1990, McCurdy and Harmon, 1992).

Inducible CDPKs

It was reported that the activity of CDPKs is induced by gibberellin (Abo-El-Saad and Wu, 1995), sugar (Ohto and Nakamura, 1995), and polyethylene glycol (Pestenacz and Erdei, 1996). The activity is also changed depending on the developmental stage of tuberization in potato (MacIntosh

et al., 1996). However, since the experiments were performed with partially purified proteins or with proteins with very low specific activity, it is hard to make any conclusion whether the changes observed in the activity of CDPK are directly related to those systems.

Several cDNAs encoding CDPK have been described to be inducible by extracellular stimuli (Botella et al., 1996, Breviario et al., 1995, Monroy and Dhindsa, 1995, Urao et al., 1994) as well as during seed development (Kawasaki et al., 1993). Botella et al. (1996) reported that a CDPK gene, VrCDPK1, cloned from IAA-induced mungbean cDNA library, was induced by mechanical strain, salt, IAA, and cycloheximide. Each stress shows different expression kinetics; maximum expression at 1 h after treatment with mechanical strain, 4 h with salt, and 6 h with IAA and cycloheximide. Two CDPK clones isolated from a cDNA library prepared from dehydrated *Arabidopsis thaliana*, were shown that these clones are inducible by drought (Urao et al., 1994). These genes are also induced by high-salt stress but not by ABA, nor by low temperature. It was suggested that a change in the osmotic potential of the environment is responsible for the induction of the genes. In addition, cold, light, and annoxia are reported to induce CDPK genes. Monroy and Dhindsa (1995) described that cold treatment induces a CDPK transcript within 3 h in alfalfa. Light and annoxia are also effective in inducing the level of CDPK transcription in rice (Breviario et al., 1995).

A recent study suggested that CDPK is a component in stress signal transduction (Sheen, 1996). By using a reporter gene with a promoter inducible by cold, salt, dark, and ABA in transient expression experiments, Sheen (1996) showed that constitutively active mutant of CDPK from *Arabidopsis*, ATCDPK1 (Urao et al., 1994) activates the promoter in the absence of extracellular stimuli. Several other CDPKs failed to produce the

same effect, indicating the response was resulted specifically by ATCDPK1. It was suggested that this CDPK is a positive regulator controlling stress signal transduction in plants. Considering that this gene itself is inducible by similar stimuli (Urao et al., 1994), this study leads to question whether there is basal activity of this CDPK present prior to stimulation or whether there is another component preceeding this CDPK in the pathway (Harmon, 1997).

In summary, CDPKs have unique structural organization. The calmodulin-like domain is fused to the catalytic domain through the junction domain. It is of interest to determine how this organization regulates the enzyme activity in the presence of calcium. It is clear that CDPKs are involved in a variety of cellular responses in plants. Further investigations that determine isoform specificities, identify and characterize endogenous substrates, and locate the subcellular compartment, are important to define better the physiological roles of CDPKs in plants.

CHAPTER 2 ACTIVATION MECHANISM OF CDPK α

Introduction

It is well established that calcium plays a role as a second messenger in diverse physiological processes in plants (Bush, 1993, Gilroy et al., 1993, Hepler and Wayne, 1985, Poovaiah and Reddy, 1993). The principal targets of calcium signals are calcium-modulated proteins, and the most well-studied calcium-modulated proteins in plants are calmodulin and calcium-dependent, calmodulin-independent protein kinase (Roberts and Harmon, 1992). Calmodulin has no intrinsic enzymatic activity, but when complexed with Ca^{2+} it binds to and modulates the activities of various enzymes. In contrast, CDPK is activated by the direct binding of Ca^{2+} to its regulatory domain that is similar in primary structure to calmodulin (Harmon et al., 1987, Harper et al., 1991, Putnam-Evans et al., 1990). This unusual structural feature places CDPK in a new class of calcium-regulated protein kinases.

CDPKs contain four domains (listed in order from the amino terminus to the carboxyl terminus): an amino terminal domain that varies in length and sequence among CDPKs and has no similarity to other proteins; a protein kinase catalytic domain most closely related in sequence to the catalytic domains of Ca^{2+} /calmodulin-dependent protein kinase family; a junction domain that functions in autoinhibition; and a calmodulin-like domain. The calmodulin-like domain (CLD) of CDPKs is 30-40% identical to calmodulin from seed plants or mammals, and it confers calcium sensitivity to the

enzyme (Harmon et al., 1994, Harper et al., 1994, Zhao et al., 1994). As is true for calmodulin, all four EF-hands of *Plasmodium* CDPK bind Ca^{2+} (Zhao et al., 1994). Both calmodulin and soybean CDPK bind to phenothiazine and naphthalene sulfonamide compounds in a calcium dependent manner (Harmon et al., 1987, Putnam-Evans, 1986, Putnam-Evans et al., 1990). These observations suggest that CLD and calmodulin have some functional similarities.

Previous studies showed that the junction domain is responsible for autoinhibition of the enzyme probably via a pseudosubstrate mechanism (Harmon et al., 1994, Harper et al., 1994). The pseudosubstrate is a sequence having features of phosphorylation sites recognized by the enzyme, but which is missing a phosphorylatable residue. This sequence interacts with the active site and blocks entry of substrate (Kemp and Pearson, 1991, Soderling, 1990). Removal of the junction and calmodulin-like domains from CDPK results in a constitutively active enzyme, while removal of the calmodulin-like domain alone results in an enzyme that is inactive (Harmon et al., 1994, Harper et al., 1994). A study with synthetic peptides showed that a peptide corresponding to residues 310-332 of the junction domain of soybean CDPK α competitively inhibited activity with respect to a peptide substrate (Harmon et al., 1994). These observations support the hypothesis that CDPKs are autoinhibited in the absence of calcium by a pseudosubstrate mechanism.

A well-documented conformational change occurs in calmodulin upon binding Ca^{2+} that allows the Ca^{2+} /CaM complex to bind to target enzymes and modulate their activities (Ikura et al., 1992). If a similar conformational change occurs in CLD in the presence of calcium, such a change could bring about activation of CDPK. Since CLD is covalently attached and is adjacent to the junction domain of CDPK, it is possible that a

conformational change could be transmitted through the peptide chain to the junction domain, and activate the enzyme (the conduit model).

Alternatively, the conformational change could lead to binding of CLD to the junction domain (the binding model) in a mechanism similar to the activation of CaMKII and MLCK by calmodulin (Soderling, 1990; Somlyo & Somlyo, 1994). It is also possible that a combination of the two mechanisms occurs.

In this work, a molecular genetic approach was used to investigate the nature of the activation of CDPK by Ca^{2+} . A chimeric CDPK in which calmodulin was substituted for the calmodulin-like domain of CDPK α , was constructed and shown to be fully active and regulated by Ca^{2+} . To determine whether activation of the CDPK α is achieved by binding between the junction domain and calmodulin-like domain, the calmodulin-like domain (CLD) was individually expressed and tested for binding and activation of a series of mutant CDPK α s in which all or part of the CLD had been deleted. The results show that binding of the CLD to the junction domain contributes to activation, but indicate that binding is not sufficient for complete activation.

Experimental Procedures

Materials

Syntide-2 (Hashimoto and Soderling, 1987) and CDPK α peptides (Harmon et al., 1994) were synthesized by the Protein Chemistry Core Laboratory at the University of Florida. Glutathione agarose and iminodiacetic acid Sepharose were purchased from Sigma. pGEX-KG (Guan and Dixon, 1991) was a gift from Dr. E. Schaller (Univ. of New Hampshire), pVUCH1 (Roberts et al., 1985) from Dr. D. Roberts (Univ. of Tennessee),

ACaM-2 from Dr. R. Zielinski (Univ. of Illinois), CaMPKII from Dr. T. Soderling (Vollum Institute, Oregon Health Sciences Center, Portland, OR) and pETH-3b from Dr. D. McCarty (Univ. of Florida). *Escherichia coli*, PR 745 was from Dr. D. Randall (Univ. of Missouri). [γ - 32 P]ATP (3000 Ci/mmol) and [γ - 32 S]dATP (250 μ Ci/mmol) were from DuPont. Restriction endonucleases, T4 DNA ligase, klenow fragment of DNA polymerase I, T4 polynucleotide kinase, calf intestinal alkaline phosphatase, Taq DNA polymerase and DNA molecular weight standards were from Gibco BRL, Promega, and New England Biolabs.

Plasmid Construction and Site-Directed Deletion Mutagenesis

Plasmids, pET1530, pHis1530 and pAY, each encoding full-length CDPK α , were described previously (Harmon et al., 1994). The integrity of cDNA inserts was verified by DNA sequencing (Sequenase version 2.0, Amersham Life Science).

Deletion mutants

Deletion mutagenesis was performed by employing expression-cassette polymerase chain reaction (PCR) (Macferrin et al., 1990) and pAY as a template. The restriction sites underlined and given names in parentheses on each primer below were to facilitate subcloning procedure and to add an initiation codon where needed.

pHis₆-CDPK α (1-328), pHis₆-CDPK α (1-333), pHis₆-CDPK α (1-339), pET-CDPK α (329-508). The numbers in parentheses indicate amino acid residues of CDPK α . A sense primer for pHis₆-CDPK α (1-328), pHis₆-CDPK α (1-333), and pHis₆-CDPK α (1-339) was GGTCCCATATGGCTGCGAAATCTAGTTCG (*NdeI*) and antisense primers were GCGCATATGTTAAATAACACGCAATGC (*NdeI*), CGCGGA TCCTTAAGATAGCCTCTCAGCAAT (*BamHI*), and

CGCGGATCCTTA TCCACCAATTTCTTCTTCTCAG (*Bam*HI), respectively. The sense and antisense primers for pET-CDPK α (329-508) were GCGCATATGGCTGAGAGGCTATCT (*Nde*I) and GCGCATATGTTATTACTTAAAGTAGCC (*Nde*I), respectively. The PCR products were digested with restriction enzymes and the resulting fragments were inserted into the corresponding sites of the expression vectors, pET-15b for His₆ constructs and pET-3b for pET-CDPK α (329-508).

pHis₆-CDPK α (1-312) and pGEX-KG/CDPK α (1-312). A sense primer was GGCCTCTAGACCATATGGCTGCGAAATCTAG (*Xba*I, *Nde*I) and an antisense primer was CGCGGATCCTTATTTCTAGACGTGATAAAAC (*Bam*HI). The expression vector used was pET-15b and pGEX-KG.

pHis₆-CDPK α (1-398). This construct was derived originally from pET1530 and made by exploiting restriction sites. One *Eco*RI site is present internally and the other is on the 3' side of the insert on the vector. Digestion with *Eco*RI followed by filling-in with klenow fragment of DNA polymerase I and ligation resulted in GAATTAATTCATCGATGA. The underlined codon encodes a stop. This manipulation gave the resulting mutant to include four extra, vector-encoded amino acid residues (LIHR) at the carboxyl terminus. This plasmid was digested with *Nde*I and *Bgl*II and the resulting fragment was inserted into *Nde*I and *Bam*HI sites of pET-15b.

Substitution mutant

For construction of pHis₆-CDPK α (1-328)-VUCH1, which encodes a CDPK α -calmodulin chimera, a cDNA insert encoding CDPK α (1-328) was amplified by PCR and ligated into an intermediate vector, pUC19. The sense and antisense primers were GGTCCCATATGGCTGCGAAATCTAGTTCG (*Nde*I) and CGCAGATCTGCAATAACACGCAATGCC (*Bgl*II). pVUCH1 encoding calmodulin was digested with *Bcl*I and *Bam*HI, and ligated to the

intermediate vector. After confirming the orientation of the insert, the resulting plasmid was cut with *NdeI* and *BamHI* and cloned into the expression vector, pET-15b.

Expression and Purification of Full-Length and Mutants of CDPK α

E. coli hosts for the expression of pEth-3b- and pET-15b-derived vectors were BL21(DE3) or BL21(DE3)pLysS (Novagen), and for pGEX-KG-derived vectors were PR 745. Protease inhibitors (1 mM PMSF, 10 μ g/ml leupeptin, and 20 μ g/ml aprotinin) were added to all the cell resuspension buffers. Extraction and purification steps were performed at 4°C. Overnight-grown cells in Luria broth (10 g Tryptone, 5 g yeast extracts, and 10 g NaCl per liter) were used to inoculate the second culture in M9TB (Studier et al., 1990). The inoculum size was 1/500. When cell density reached 0.4 - 0.5 at OD₆₀₀, the culture was cooled to room temperature. IPTG was added to 0.5 mM to induce the expression and the cells were further cultured for 3 h at room temperature (20 - 25 °C). Harvested cells were stored at -80 °C.

For the purification of recombinant His₆-fusion proteins, cells were thawed and resuspended in binding buffer (20 mM Tris, pH 8.0, 0.5 M NaCl, 5 mM imidazole), sonicated, and cellular debris was pelleted by centrifugation. The supernatant was made 1% (v/v) in Triton X-100 and filtered through a 0.2 μ m filter. The filtrate was loaded onto a nickel chelation column prepared from iminodiacetic acid-Sepharose and connected to an FPLC (Pharmacia). The column was washed with 10 column volumes of wash buffer (20 mM Tris, pH 8.0, 0.5 M NaCl, 60 mM imidazole) and eluted with elution buffer (20 mM Tris, pH 8.0, 0.5 M NaCl, 400 mM imidazole). Fractions containing expressed proteins were pooled and dialyzed overnight with three, 4 L changes of Mono Q equilibration buffer (20 mM Tris, pH 8.0, 2.5 mM EDTA).

Dialyzed samples were loaded onto Mono Q, HR 5/5 column (Pharmacia) and eluted with a gradient of 0 - 0.5 M NaCl in equilibration buffer. Fractions of the highest purity as determined by SDS-PAGE were pooled and concentrated by ultrafiltration (Centricon 10, Amicon). The buffer of concentrated proteins were changed with 20 mM Tris, pH 8.0 and 0.1 mM DTT while in the concentration device. Glycerol was added to the final concentration of 10% or 50% (v/v) and the enzyme was stored at - 80 or -20 °C.

Cells expressing pGEX-KG/CDPK α (1-312) were treated as above except that the cell resuspension buffer (buffer A) was 50 mM Tris, pH 8.0 and 150 mM NaCl. The extract was loaded onto a glutathione-agarose column equilibrated in buffer A. After extensive washing with buffer A, bound proteins were eluted with 50 mM Tris, pH 7.2 and 10 mM reduced glutathione. Fractions containing kinase activity were pooled and chromatographed on blue-Sepharose as previously described (Putnam-Evans et al., 1990).

Recombinant calmodulin-like domain (CLD) was purified by a modification of methods used for the purification of calmodulin (Anderson, 1983, Roberts et al., 1985). Cells expressing CLD were resuspended in lysis buffer (50 mM Tris, pH 7.2, 0.5 M NaCl, 2 mM EDTA, 1 mM DTT) and sonicated. Clarified cell extracts were boiled for 5 min and denatured proteins were pelleted by centrifugation. The supernatant was made 5 mM in CaCl₂ and was loaded on phenyl-Sepharose equilibrated with 20 mM Tris, pH 7.2 and 1 mM CaCl₂. After extensive washing with 20 mM Tris, pH 7.2 and 0.5 mM CaCl₂, CLD was eluted with 20 mM Tris, pH 7.2 and 2.5 mM EDTA. Pooled fractions from phenyl-Sepharose were applied to Mono Q HR 5/5 equilibrated with 20 mM Tris, pH 7.2 and 2.5 mM EDTA. Proteins were eluted with a gradient 0 - 0.5 M NaCl in the same buffer. Fractions containing CLD

were dialyzed extensively against 5 mM NH_4HCO_3 , then against 20 mM Tris, pH 7.2 and 0.1 mM DTT.

Biotinylation of Calmodulin-like Domain and Overlay

CLD was biotinylated as described by Billingsley et al. (1985) with Biotin-X-NHS (Calbiochem) in the presence of 1 mM CaCl_2 . For the overlay assay, proteins were separated by electrophoresis in SDS-polyacrylamide gels in the presence of 1 mM EGTA and electroblotted to nitrocellulose. Blots were blocked in 5% (w/v) non-fat dry milk in TBS (50 mM Tris, pH 7.5 and 300 mM NaCl) for 1 h at room temperature with constant agitation and washed in TBS for 10 min. Blots were incubated in TBS containing 1 % (w/v) gelatin with biotinylated CLD (5 $\mu\text{g}/\text{ml}$) for 2 h in the presence of 1 mM CaCl_2 and then washed twice with TBS plus 1 mM CaCl_2 . Biotinylated CLD was detected with ExtrAvidin®-alkaline phosphatase conjugate (Sigma) at a 1:1,000 dilution. Control experiments contained 5 mM EGTA in place of CaCl_2 .

Protein Kinase Assays

Activity assays were performed as described by Harmon, et al. (1994) with the following modification. The assay buffer contained 50 mM HEPES, pH 7.2, 10 mM MgCl_2 , 1 mM EGTA, 0.4 mg/ml BSA, with or without 1.1 mM CaCl_2 . Truncated $\text{CDPK}\alpha$ s were assayed in a volume of 25 μl with 200 μM syntide-2 (peptide substrate), 20 nM recombinant enzyme and the indicated concentrations of CLD. BSA was added in place of CLD to keep the total protein concentration constant in all tubes. The dependence of the activity of His₆- $\text{CDPK}\alpha$ (1-398) on the concentration of CLD was done as above except with 100 μM syntide-2, 1 mM DTT, and 1 mg/ml BSA. The activity of recombinant calcium/calmodulin dependent protein kinase II was

determined as described in Harmon, et al. (1994) with 2 μM or 20 μM CLD and with ACaM-2 as a control.

To facilitate comparison of activities of proteins of different sizes, activities are reported on a molar basis ($\mu\text{mol min}^{-1} \mu\text{mol}^{-1} = \text{min}^{-1}$). Moles of enzymes were calculated from molecular weights predicted from cDNA sequences: His₆-CDPK α , 59,339; His₆-CDPK α (1-398), 47,149; His₆-CDPK α (1-339), 40,403; His₆-CDPK α (1-333), 39,788; His₆-CDPK α (1-328), 39,232; GST-CDPK α (1-312), 63,160; CLD, 21,124; ACaM-2, 16,689.

Tryptic Digestion

CDPK α and truncation mutants (0.2 mg/ml) were digested with 10 $\mu\text{g/ml}$ trypsin (TPCK-treated, Worthington) in buffer containing 1 mM EGTA, 30 mM NaCl and 30 mM Tris, pH 7.5 at 25 °C. At various times an aliquot was removed and added to 100 $\mu\text{g/ml}$ soybean trypsin inhibitor to terminate digestion. Activity of digested enzyme was determined as described above.

Electrophoresis

SDS-PAGE was conducted according to the method of Laemmli (1970). Glycerol PAGE was performed as described in Persechini, et al. (1986) with the following modifications. Samples were prepared by combining calmodulin-like domain (100 pmol) with 100 or 1000 pmol of indicated CDPK α peptides in the presence of 1 mM CaCl₂ or 2 mM EGTA. Samples were electrophoresed at 300 V and 10 °C for 28 - 48 h in 1.5 mm polyacrylamide slabs (16 x 18 cm) containing 10% (w/v) acrylamide, 0.5% (w/v) bisacrylamide, 40% (w/v) glycerol, 20 mM Tris, pH 8.6, 23 mM glycine, and with either 1 mM CaCl₂ or 2 mM EGTA. Pre-electrophoresis was performed at 400 V and 10 °C for 1.5 h. The reservoir buffers contained 20 mM Tris, pH 8.6, 23 mM glycine, and with

either 1 mM CaCl₂ or 2 mM EGTA. The reservoir buffers were refurnished several times during electrophoresis. Urea/glycerol gel electrophoresis was carried out as above except 4M urea was added to the polyacrylamide gel and samples.

Other Procedures

The concentration of syntide-2 was determined from amino acid composition analysis done in the Protein Sequencing Core Facility, University of Florida. The concentrations of recombinant proteins were measured by the Bio-Rad dye-binding assay, based on the method of Bradford (Bradford, 1976). Concentrations of CLD and *Arabidopsis* CaM-2 were estimated with the method of Lowry et al. (1951). Digital images of polyacrylamide gels and nitrocellulose blots were obtained as previously described (Harmon et al., 1994). Oligonucleotides were synthesized at the DNA Synthesis Core Laboratory, University of Florida.

Results

CDPK α -Calmodulin Chimera is Similar to CDPK α in Activity

To assess whether calmodulin is able to function in place of CDPK α 's CLD, a chimeric enzyme was made in which CLD was replaced by a synthetic CaM, VU-1. The sequence of VU-1 is a conservative hybrid between plant and vertebrate CaM, and the protein is functionally comparable to CaM (Roberts et al., 1985). The chimeric enzyme contained the first 328 amino acids of CDPK α (the amino-terminal, catalytic, and junction domains), but CLD (the carboxyl-terminal 180 residues of CDPK α , ³²⁹AERL...GYFK₅₀₈) was replaced by the complete VU-1 sequence (¹ADQL...MMAK₁₄₈). The number of residues

separating the junction domain and the first EF hand was conserved between the chimeric enzyme and CDPK α (Fig. 2-1).

Both CDPK α and the chimera were expressed as His₆-tagged fusion proteins and purified by metal chelation chromatography. Since a high degree of purification was essential in order to assess accurately the effect of the substituted sequence, the proteins were purified further on Mono Q HR 5/5 (A and B in Fig. 2-1). Both proteins were eluted at 200 mM NaCl.

The activities of CDPK α and the chimera were stimulated 30 - 70 fold by calcium, and both enzymes had similar values of apparent $V_{\max}$ (Table 2-1). The apparent K_M for syntide-2 was slightly higher for the chimera than for CDPK α (Table 2-1). These results show that calmodulin can mimic the properties of CLD in transducing the calcium signal to activate fully the catalytic activity of CDPK. While these results suggest that CLD and VU-1 may use a mechanism involving Ca²⁺-dependent conformational changes to activate the protein kinase, they do not reveal what the mechanism is. We undertook the following work to ask if activation results from the binding of the CLD to the junction domain of CDPK α in a manner analogous to the activation of calmodulin-dependent enzymes by calmodulin.

Table 2-1: Kinetic Parameters of CDPK α and the Chimeric Enzyme, CDPK α (1-328)-VU-1

Enzyme	$K_{m_{app}}$ (μ M)	$V_{max_{app}}$ (μ mol/min/mg)
CDPK α	24.4, 28.6	3.19, 3.46
CDPK α (1-328)-VU-1	37.8, 49.5	3.60, 3.93

Kinetic parameters for enzymes were determined from double reciprocal analysis of data obtained at an ATP concentration of 60 μ M and various concentration of syntide-2 as peptide substrate. Results are reported from two independent experiments.

Figure 2-1: Structural features of recombinant proteins used in this study.

Unmodified CDPK α was expressed as fusion protein with an affinity tag containing six histidine residues (His₆) at the amino terminus. The domains of CDPK α are labeled as follows: amino-terminal domain of undefined function (U), catalytic domain (Cat), junction domain (J), calmodulin-like domain (CLD). Residue numbers marking the boundaries of domains are indicated below the molecular diagram. The chimeric enzyme has the same structure as CDPK α except that synthetic calmodulin (VU-1) replaces CLD. The Δ CLD mutants were produced by deletion of all or part of the CLD. The constitutively active mutant GST-CDPK α (1-312), which contains the amino terminal and catalytic domains plus a few residues of the junction domain, was expressed as a fusion protein with glutathione S-transferase (GST) at the amino terminus. CLD (residues 329-508 of CDPK α) was not expressed as a fusion protein.

Full Name	Descriptive Name	Diagram
His ₆ -CDPK α	Full length	
His ₆ -CDPK α (1-328)-VUCH1	Chimera	
His ₆ -CDPK α (1-398)	Δ 52%CLD	
His ₆ -CDPK α (1-339)	Δ 92%CLD	
His ₆ -CDPK α (1-333)	Δ 97%CLD	
His ₆ -CDPK α (1-328)	Δ 100%CLD	
GST-CDPK α (1-312)	Catalytic domain	
His ₆ -CDPK α (329-508)	CLD	

Expression, Purification, and Properties of Truncation Mutants

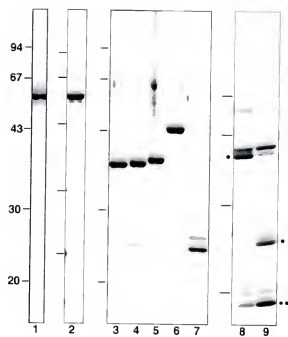
If binding of CLD to the junction domain is the major event that activates the enzyme, then CLD should not have to be covalently attached to the rest of the enzyme in order to accomplish activation. In preparation for testing this prediction, several truncation mutants (Fig. 2-1) were expressed, purified, and characterized.

Expression vectors that produce either N-terminal His₆-tagged or N-terminal GST-tagged fusion proteins were used. The His₆-tagged proteins were purified by metal chelation and anion exchange chromatography. His₆-CDPK α (1-398) was eluted from Mono Q HR 5/5 with a NaCl gradient, but His₆-CDPK α (1-328), (1-333), and (1-339) were recovered in the flow-through fractions. The chromatographic behavior of the latter three proteins corresponded to the deletion of the acidic CaM-like domain (calculated pI = 4.2). The purified recombinant enzymes are shown in Fig. 2-2.

Purification of the catalytic domain (residues 1-312) of CDPK α proved to be more difficult. Unlike the other His₆-proteins, His₆-CDPK α (1-312) did not bind to the metal chelation column in the presence of 60 mM imidazole. Also, when this protein was applied to the hydrophobic chromatography resin, phenyl-Sepharose, it could not be eluted unless 6 M urea was employed. However, expression of the catalytic domain as a GST fusion protein allowed its purification by affinity chromatography on glutathione agarose followed by chromatography on blue-Sepharose, a resin useful for the purification of CDPK (Putnam-Evans et al., 1990) and other protein kinases (Jeno and Thomas, 1991). Unlike native CDPK, GST-CDPK α (1-312) was not effectively eluted from this resin by 0.2% (w/v) CHAPS, and only 5% of the

Figure 2-2: Purity of recombinant proteins.

Purified recombinant proteins (2 μ g) were analyzed by electrophoresis in 12% polyacrylamide gel in the presence of SDS, which was stained with Coomassie blue. Lane 1, His₆-CDPK α ; 2, His₆-CDPK α (1-328)-VU-1; 3, His₆-CDPK α (1-328); 4, His₆-CDPK α (1-333); 5, His₆-CDPK α (1-339); 6, His₆-CDPK α (1-398); 7, CLD; 8, GST-CDPK α (1-312); 9, GST-CDPK α (1-312) treated with thrombin for 50 min. The dot to the left of lane 8 indicates the position of GST-CDPK α (1-312). The positions of CDPK α (1-312), and GST in lane 9 are indicated by single and double dots, respectively. The positions of molecular mass markers are indicated by the lines to the left of each panel. From top to bottom they are: phosphorylase b, 94 kDa; BSA, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa.



applied activity was recovered. These observations suggest that the CDPK catalytic domain is more hydrophobic in nature than the full length enzyme. The specific activity of GST-CDPK α (1-312), which contains the catalytic domain plus 14 residues of the junction domain, was 0.65 $\mu\text{mol}/\text{min}/\text{mg}$ (41 min^{-1} or 24% of the activity of full length CDPK α). As reported previously (Harmon et al., 1994, Harper et al., 1994), its activity was not affected by either the presence or absence of calcium. The low specific activity was due in part to the presence of other proteins in the prep (see lane 8 in Fig. 2-2), some of which might be derived proteolytically from GST-CDPK α (1-312). Other factors contributing to the low specific activity could be the hydrophobic nature of this mutant enzyme, the presence of some of the residues of the junction domain, and/or the inherent instability of the truncated enzyme.

CLD was not expressed as a fusion protein (see Experimental Procedures), because it could be purified successfully by methods (heat treatment and calcium-dependent chromatography on phenyl-Sepharose) used for calmodulin purification. Preparations of purified CLD contained two polypeptides observed in SDS-PAGE (lane 7 in Fig. 2-2). Both polypeptides showed a calcium-dependent mobility shift on gel electrophoresis and could bind phenyl-Sepharose in a calcium-dependent manner (data not shown), therefore both components of CLD are functional. Analysis of recombinant CLD by mass spectrometry revealed that it is proteolytically cleaved during expression between Arg483 and Lys484 (Jorg Heierhorst and Bruce Kemp, personal communication).

Calmodulin-like Domain Binds to ΔCLD Mutants

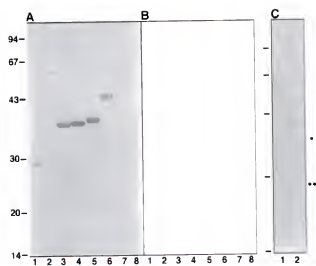
If activation of CDPK involves the binding of the CLD to the junction domain, then CLD should bind to CDPK mutants that contain the junction

domain. To test this prediction, biotinylated CLD was incubated with nitrocellulose blots of full-length and truncated CDPK in the presence (Fig. 2-3A) or absence (Fig. 2-3B) of calcium. The background staining of the blots incubated with calcium was high relative to that of the blot incubated without calcium, regardless of whether non-fat milk, BSA, or gelatin was used for blocking the membrane. The strongest signals were observed in lanes containing His₆-tagged truncation mutants (Δ CLD) ending at residues 328, 333, 339, and 398, all of which contain the junction domain (lanes 3 - 6, Fig 2-3A). Binding of biotin-CLD to these proteins was greatly enhanced in the presence of calcium (compare Fig. 2-3A and B). Weak staining of both GST-CDPK α (1-312) (lane 2) and GST (lane 1) was observed. GST-CDPK α (1-312) was cleaved by digestion with thrombin into GST and CDPK α (1-312) (Fig. 2-2, lanes 8 and 9), and very weak staining of both polypeptide products was observed (Fig. 2-3C). Biotin-CLD did not bind to the calmodulin-like domain (Fig. 2-3, lanes 8), which contains residues 329-508. These results confirm that a CLD-binding site is not located in the calmodulin-like domain. In addition this observation suggests that there is no intermolecular interaction between CLDs of separate molecules of CDPK α . Taken together these results show that a binding site for CLD is present in the junction domain between residues 312 and 328.

Although biotin-CLD bound to Δ CLD mutants of CDPK α , it bound only weakly to full-length enzyme (lanes 7 in Fig. 2-3). This observation agrees with previous experiments in which biotinylated soybean calmodulin failed to bind full length CDPK (Harmon et al., 1994). In the presence of calcium, the binding site for CLD may not be accessible to biotin-CLD due to steric hindrance and/or occupation of the binding site by the intrinsic calmodulin-like domain. The observation that biotin-CLD is unable to bind full length

Figure 2-3: Binding of biotinylated CLD by full length and truncated CDPK α .

Proteins (2 μ g) were resolved by electrophoresis in a 12% SDS-polyacrylamide gel the presence of 1 mM EGTA and blotted onto nitrocellulose. The blots were overlaid with biotinylated CLD in the presence of Ca²⁺ (A and C) or EGTA (B) as described in Experimental Procedures. A and B: Lane 1, GST; 2, GST-CDPK α (1-312); 3, His₆-CDPK α (1-328); 4, His₆-CDPK α (1-333); 5, His₆-CDPK α (1-339); 6, His₆-CDPK α (1-398); 7, His₆-CDPK α ; 8, CLD. C: Lane 1, GST-CDPK α (1-312); GST-CDPK α (1-312) treated with thrombin for 50 min. The positions of CDPK α (1-312), and GST in lane 2 are indicated by single and double dots, respectively. The molecular mass markers are the same as those described in the legend to Fig. 2-2.



CDPK supports the hypothesis that binding of the CLD to the junction domain is intramolecular and not intermolecular. This hypothesis is further supported by the observations that native soybean CDPK (Putnam-Evans et al., 1990) and recombinant CDPK α (data not shown) are monomeric enzymes that do not dimerize in the presence of calcium.

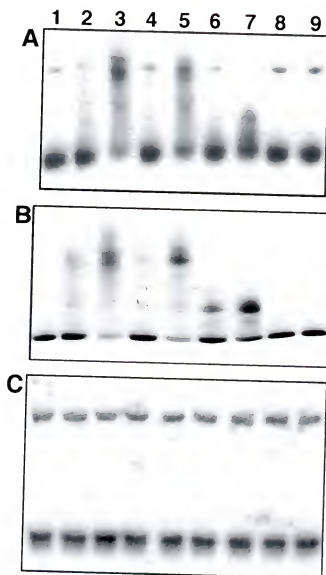
CLD Forms Complexes with Junction Domain Peptides

To examine further the binding of CLD to the junction domain, synthetic peptides corresponding to the junction domain were mixed with CLD and subjected to electrophoresis in non-denaturing glycerol polyacrylamide gels in the presence and absence of 4 M urea. The positively charged synthetic peptides migrated toward the cathode and did not enter the gel (data not shown). Two bands were observed in lanes containing CLD (predicted pI = 4.2 and MW = 20,124) (lanes 1, Fig. 2-4). The slower migrating band was an artifact due to oxidation of the CLD, since this band was not present in samples treated with 10 mM DTT (data not shown). Complexes of CLD and basic peptides had mobilities different from either of the two CLD species.

In the presence of Ca²⁺, the mobility of CLD was not greatly affected by equimolar (100 pmol) CDPK α (302-332), (310-32) or (318-32) (Fig. 2-4A, lanes 2, 4, 6), but a ten-fold molar ratio of the peptides to CLD resulted in the appearance of bands corresponding to complexes and the concomitant diminution of the CLD bands (Fig. 2-4A, lanes 3, 5, 7). Inclusion of 4 M urea in the gel did not alter these results (Fig. 2-4B). The greater mobility of the CLD•CDPK α (318-32) complex (Fig. 2-4A and B, lane 7) relative to that of complexes with the other two peptides (lanes 3, 5) can be attributed to the lower content of basic amino acids in this peptide relative to the others. A

Figure 2-4: Complex formation between CLD and synthetic peptides.

CLD (100 pmol, 2 μ g) was electrophoresed in nondenaturing gels alone (lane 1) or in the presence of 100 pmol (even lanes) or 1000 pmol (odd lanes) of each of the following peptides: lanes 2 and 3, CDPK α (302-32); lanes 4 and 5, CDPK α (310-32); lanes 6 and 7, CDPK α (318-32); lanes 8 and 9, CDPK α (302-17). Panel A: CaCl₂ (1 mM) was added to all samples and gel buffers, and samples were analyzed by electrophoresis in a glycerol gel. Panel B: Same as A, except 4M urea was added to all samples and buffers. Panel C: Samples were analyzed by electrophoresis in the presence of 2 mM EGTA and 4 M urea.



fourth peptide, CDPK α (302-317), corresponding to residues in the amino-terminal region of the junction domain, did not form a complex with CLD at either 1:1 or 10:1 molar ratio (Fig. 4A and B, lanes 8 and 9). Taken together, these results show that a minimal binding site for CLD is located between residues 318 and 332. These results are in agreement with the biotin-CLD blot overlay experiment (Fig. 2-3) which locates the CLD binding site between residues 312 and 328.

To test whether complex formation was dependent on calcium, mixtures of CLD and peptides were examined in gels containing EGTA. When urea was omitted from the gels, CLD and peptides formed aggregates that did not migrate into the gel (data not shown). Addition of 4 M urea prevented aggregation and allowed entry of the protein into the gel. There was no difference between the pattern of migration in lanes containing CLD alone and lanes containing mixtures of CLD and peptides (Fig. 2-4C). Comparison of these results with those in Fig. 2-4B shows that binding of the peptides to CLD is dependent on the presence of calcium.

Added CLD Reverses Inhibition of Constitutively Active CDPK by Peptide 310-332

GST-CDPK α (1-312) contains the catalytic domain and a few residues of the junction domain, and it is active in the presence and absence of calcium (Harmon et al., 1994). Consistent with the hypothesis that CDPK is autoinhibited by a pseudosubstrate sequence located in the junction domain, GST-CDPK α (1-312) is inhibited by peptide CDPK α (310-332) which corresponds to the carboxyl end of the junction domain (Fig. 2-5). The inhibition is competitive with respect to syntide-2 and the K_i is 5 μ M (Harmon et al., 1994). Since a CLD-binding site is also located between residues 310-332 (Fig. 2-4), we

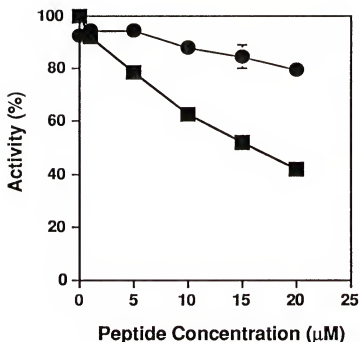


Figure 2-5: Effect of CLD on inhibition of GST-CDPK α (1-312) by peptide 310-332.

The activity of GST-CDPK α (1-312) assayed in a mixture containing 15 μM BSA and 100 μM syntide-2 in the presence of calcium was $0.43 \mu\text{mol min}^{-1} \text{mg}^{-1}$. Activity was measured in the presence (closed circles) or absence (closed squares) of 20 μM CLD and various concentrations of CDPK α (310-332). Data points are the mean and standard deviation of determinations from two independent experiments.

asked if inhibition of CDPK α (1-312) by peptide CDPK α (310-332) could be reversed by CLD. Addition of 20 μ M CLD to an activity assay in which the concentration of inhibitory peptide was varied protected the enzyme from inhibition (Fig. 2-5). The results suggest that binding of CLD and the catalytic domain to peptide 310-332 is mutually exclusive and support the binding model of activation.

CLD Activates Δ CLD Mutants

To test whether CLD can activate CDPK α in trans, the effect of adding CLD to four Δ CLD mutants in the presence and absence of calcium was determined. Since the exact location of the CLD binding site was unknown, three mutants terminating at residues 328, 333, and 339 near the interface between the junction and calmodulin-like domains were tested. The fourth mutant terminated at residue 398 and contained one complete and one partial EF-hand. To control for nonspecific effects of protein concentration in these assays, the total protein was held constant by addition of BSA. In the absence of CLD the activities of His₆-CDPK α (1-328), (1-333), (1-339), and (1-398) were low both in the presence and absence of Ca²⁺ but full length CDPK α was activated 44-fold by Ca²⁺ (Table 2-2, -CLD). These results are consistent with previous studies showing that the junction domain (residues 302-332) functions as an autoinhibitory domain, (Harmon et al., 1994, Harper et al., 1994). Furthermore, they show that the presence of most of two EF hands is not sufficient for activation of the enzyme by Ca²⁺. Addition of 20 μ M CLD to full length CDPK α in the presence or absence of calcium had no effect on activity (Table 2-2). With the exception of one trial with CDPK α (1-333), addition of CLD in the absence of calcium to the Δ CLD mutants stimulated activity $\leq$ 2-fold, while addition of CLD in the presence of Ca²⁺ stimulated

Table 2-2: Effect of CLD on the activity of full-length and mutant CDPK α

Enzyme	-Ca ²⁺		+ Ca ²⁺	
	-CLD	+CLD	-CLD	+CLD
	(min ⁻¹) ^a			
His ₆ -CDPK α	4.2	3.6	157	157
His ₆ -CDPK α (1-398)	1.9	2.4	0.80	54
His ₆ -CDPK α (1-339)	0.43	0.81	0.60	5.7
	nd ^b	0.32	nd	2.8
His ₆ -CDPK α (1-333)	0.76	1.5	0.61	4.4
	0.16	0.80	0.12	8.9
His ₆ -CDPK α (1-328)	0.39	0.51	0.23	1.2

^aSpecific activities are reported on a molar basis.

Activities were determined at a syntide-2 concentration of 200 μ M, in the presence of 20 μ M CLD (+CLD) or 20 μ M BSA (-CLD), and in the presence or absence of Ca²⁺. Values in each row are for a single enzyme preparation.

^bNot determined.

activity 5 to 73-fold (Table 2-2). While the three shortest mutant CDPK α s achieved only 1 - 8% of the specific activity of full-length enzyme, each was stimulated at least 5-fold by the addition of CLD. In experiments with different preparations, His₆-CDPK α (1-398) was consistently activated to a greater extent by addition of CLD, than were the mutants in which all four EF hands were deleted (data not shown). In the presence of 20 μ M CLD and Ca²⁺, the specific activity of His₆-CDPK α (1-398) was 40% of that of full length enzyme.

To examine whether CLD was unable to activate the Δ CLD mutants because they were inherently inactive, we asked if the mutants could be activated by an alternate means. Removal of the junction domain, which is rich in basic residues, by digestion with trypsin should result in an enzyme that is active in the absence of calcium. His₆-CDPK α (1-328), His₆-CDPK α (1-398), and full length enzyme were digested with trypsin for various times, and their activities were measured in the absence of calcium (Fig. 2-6). All three enzymes had little activity at time zero, but their activities increased during digestion and then leveled off after 20 min. Examination of the digested enzymes by SDS-PAGE showed that all three enzymes were converted to single species of 34 kDa after 20 min, and this protein was resistant to further digestion (data not shown). Digested His₆-CDPK α (1-398) and full length CDPK had $\leq$ 6% of the activity of undigested enzymes measured under optimal conditions. His₆-CDPK α (1-328) was activated by digestion to the same degree as His₆-CDPK α (1-398), and this level of activity was higher than that obtained by addition of the CLD to this His₆-CDPK α (1-328) (Table 2-2). These experiments show that although His₆-CDPK α (1-328) was capable of activity, CLD was unable to fully stimulate its activity.

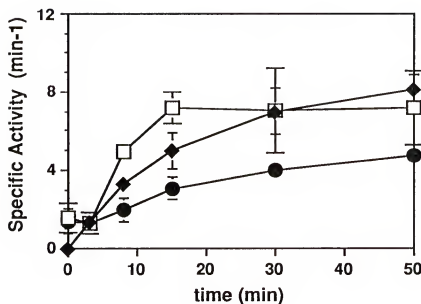


Figure 2-6: Activation of CDPK α and truncation mutants by digestion with trypsin.

CDPK α (closed circles) His₆-CDPK α (1-398) (open squares) or His₆-CDPK α (1-328) (closed diamonds) were digested with trypsin as described in Experimental Procedures. Aliquots were removed during the time course of digestion and the activity in the absence of calcium was determined. Data points are the mean and standard deviation of determinations from two independent experiments.

Calmodulin is not as Effective as CLD in Activation of His₆-CDPK α (1-398)

The dependence of the activity of His₆-CDPK α (1-398) on the concentration of CLD or *Arabidopsis* calmodulin was determined (Fig. 2-7). At 100 μ M CLD the truncated enzyme was activated 480-fold to a specific activity of 4.8 μ mol/min/mg, which when compared on a molar basis is 108% of that of full-length enzyme measured in the same conditions. The $K_{0.5}$ for CLD was 7 μ M. The high specific activity shown in Fig. 2-7 can be attributed to improved conditions, since when the experiment was performed with the same assay conditions and CLD preparation used for the experiments in Table 2-2, the maximal activity was 50% (molar basis) of that of full length enzyme and the $K_{0.5}$ was 12 μ M (data not shown). No increase in the activation of His₆-CDPK α (1-328) by CLD was observed when the improved conditions were used. The results in Table 2-2 and Fig. 2-7 show that the presence of a partial CLD sequence in His₆-CDPK α (1-398) contributes to the ability of added CLD to stimulate activity.

Calmodulin was much less effective than CLD in activation of His₆-CDPK α (1-398). Activity was stimulated 19-fold to a maximal specific activity of 0.19 μ mol/min/mg (Fig. 2-7). The $K_{0.5}$ was 20 μ M. Similar results were obtained when VU-1 calmodulin was used in place of *Arabidopsis* calmodulin (data not shown). These results suggest that activation of CDPK α and the CDPK α -VU1 chimera occurs by a mechanism other than simple binding.

Discussion

In this work, we explored whether intramolecular binding of the calmodulin-like domain to the junction domain of soybean CDPK α

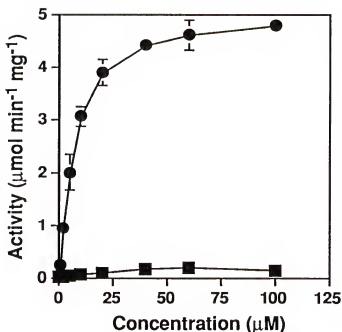


Figure 2-7: Activation of His₆-CDPKα(1-398) by addition of CLD or calmodulin.

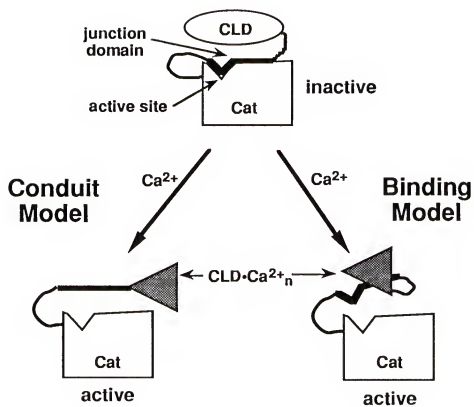
CLD (circles) or *Arabidopsis* calmodulin (squares) was added to 20 nM His₆-CDPKα(1-398) and the activity of was determined in the presence of calcium as described in Experimental Procedures except that the concentrations of BSA and syntide-2 were 1 mg/ml (15 μM) and 100 μM, respectively. The activity of full length enzyme in these conditions was 201 min⁻¹. The inset shows the data for CLD on an expanded scale. Data are the mean and standard deviations of determinations from two independent experiments.

contributes to activation. The binding model as described in Fig. 2-8 predicts: 1) CLD binds to a site in the junction domain in a calcium-dependent manner and 2) binding of CLD to the junction domain in the presence of calcium leads to activation of the enzyme. In this model the single event that leads to activation is binding between the CLD and the junction domain, therefore binding and activation should be possible whether or not CLD is attached to the rest of the molecule.

The first prediction was tested by blot overlays of truncation mutants of CDPK α . Biotinylated CLD bound to mutant enzymes containing the junction domain, but it did not bind to CLD on the blot (Fig. 2-3). Our results also show that it bound weakly to the catalytic domain and to GST, and it caused high background staining of blots incubated in the presence of calcium. The data in Fig. 2-5 show that activity of the catalytic domain in the absence of inhibitory peptide was not affected greatly by the addition of CLD. These results suggest that the weak binding of biotin-CLD to the catalytic domain is nonspecific and inconsequential. Binding of CLD to the junction domain was further examined in experiments with synthetic peptide analogs of the junction domain of CDPK α (Fig. 2-4). CLD bound to synthetic peptides containing residues 318-332, but not to the peptide containing residues 302-317. The lack of binding between the latter peptide, which has a pI of 9.0, shows that complex formation of CLD with the other peptides is not nonspecific interaction of the basic peptides with the acidic CLD. These data and the results of the blot overlay experiments show that a CLD binding site is located between residues 312 and 328 in the junction domain and fulfills the first prediction of the binding model. The observations of Huang et al., (submitted) with AK1, a CDPK from *Arabidopsis*, are in agreement with our

Figure 2-8: Models for regulation of CDPK activity.

In both models CDPK is autoinhibited in the absence of Ca^{2+} by the interaction of a pseudosubstrate sequence (V-shaped portion of the junction domain) with the active site. Activation occurs when the binding of Ca^{2+} to the calmodulin-like domain causes a conformational change in the CLD. In the conduit model (left arrow), the conformational change in the CLD affects the conformation of the adjacent junction domain and pseudosubstrate. In the intramolecular binding model (right arrow) the conformational change in the CLD allows it to bind to a binding site located in the junction domain.



findings. They have shown that the calmodulin-like domain binds to the junction domain with a K_d of 6-7 μ M.

Since CLD can bind to a site in the junction domain, the question of whether their interaction is inter- or intramolecular arises. Gel filtration chromatography performed in the presence or absence of calcium shows that active native soybean cell CDPK (Putnam Evans, 1990) and recombinant CDPK α (data not shown) are monomeric. In addition, isolated CLD does not bind to full length CDPK α (Fig. 2-3). These observations indicate that intermolecular binding of CLD to the junction domain does not occur and supports the idea that the binding is intramolecular.

To test the second prediction of the model, we measured the activity of the truncation mutants in the presence and absence of added CLD in the presence and absence of calcium. Each of the Δ CLD mutants contained all of the junction domain and in the absence of CLD all were inactive regardless of the calcium concentration. Addition of CLD in the presence of calcium stimulated the activity of all the mutants, but to varying degrees (Table 2-2). Although CLD could bind to all the Δ CLD mutants (Fig. 2-3), it did not highly activate mutant enzymes missing all of the calmodulin-like domain. Since the truncated enzymes could be activated by treatment with trypsin, the low degree of activation by CLD was not due to the inherent inactivity of the mutants. These data suggest that binding of CLD to the junction domain is not sufficient for complete activation.

Added CLD highly activated the truncation mutant His₆-CDPK α (1-398) that retained nearly half of the CLD. Neither calcium alone nor CLD alone activated this mutant, but CLD plus calcium activated it up to 450-fold (Fig. 2-7). It is possible that the conformation of the junction domain is different in His₆-CDPK α (1-398) than in the enzymes terminating at residues 298, 333, or

339. This difference in conformation could arise from the presence of additional polypeptide at the carboxyl-terminal end of the CLD-binding site, or it could be caused by a conformational change in the EF-hand(s) present in His₆-CDPK α (1-398). A conformational change in the junction domain could either promote the binding of CLD or weaken the binding of the pseudosubstrate to the active site. An alternative possibility is that the CLD binding site, which would be located at the carboxyl-terminus of the shorter Δ CLD mutants, could be subject to proteolysis during expression. The latter possibility is unlikely because the mutant enzymes migrated at positions in SDS-PAGE consistent with their predicted sizes, and all of them bound biotin-CLD (Fig. 2-3).

Half maximal activation of His₆-CDPK α (1-398) occurred at a CLD concentration of 7 μ M (Fig. 2-7) and this $K_{0.5}$ is in agreement with the K_d of 6-7 μ M for the binding of CLD and the junction domain determined by Huang et al. (1996). These data show that CLD has a lower affinity for its binding site in CDPK than does calmodulin for its binding sites in target enzymes. The binding constant of CaM with NAD kinase or MLCK is ≤ 1 nM, and is 25 to 100 nM with CaMPKII (Hanson and Schulman, 1992, Roberts and Harmon, 1992). The apparent low affinity of CLD for binding the junction domain does not preclude binding from contributing to activation, however, since the CLD is covalently attached to CDPK, and the low binding affinity may be overcome by the high local concentration of each component.

The sequences of calmodulin and CLD are only 40% identical and CLD is unable to activate the calmodulin-dependent enzymes, cyclic nucleotide phosphodiesterase (PDE), (Harmon et al., 1996) and CaMKII (data not shown). CLD does not prevent the activation of PDE by calmodulin, suggesting that CLD does not bind to this enzyme (Harmon et al., 1996). In contrast to these

observed functional differences, calmodulin can substitute for CLD in the chimeric enzyme CDPK α -VU-1, and support full, calcium-dependent activity (Table 2-1). Since both CLD (Fig. 2-3 and 4) and CaM (Harmon et al., 1996) bind to the junction domain peptides, it is possible that this is the property that allows both CLD and CaM to activate CDPK α and the chimera. However, CLD, but not calmodulin, significantly activated His₆-CDPK α (1-398) (Fig. 2-7). If the binding model (Fig. 2-8) were correct, then both CLD and CaM should activate His₆-CDPK α (1-398). The paradox that calmodulin is able to bind to the junction domain and to function in the activation of the chimera, but unable to activate His₆-CDPK α (1-398) suggests that the simple binding model is not valid.

Our data suggest that intramolecular binding between the calmodulin-like domain and a binding site in the junction domain occurs, but that binding is not sufficient for activation of CDPK. Interaction between CLD and additional parts of the enzyme may contribute to activation. Further experiments with the chimera and mutant enzymes employing site-directed mutagenesis should give insight into the mechanism, but definitive proof will most likely require the determination of crystallographic structures of CDPK.

CHAPTER 3 IDENTIFICATION AND CHARACTERIZATION OF INTERACTING PROTEINS OF CDPK

Introduction

Transient changes in intracellular free calcium concentration have been connected to many signal transduction pathways in response to a number of physiological stimuli, including light, touch, wind, pathogenic elicitors, cold-shock, gravity, and hormones such as abscisic acid, auxin, cytokinins, ethylene, and gibberellic acid (Bush, 1993, 1995, Gilroy et al., 1993, Poovaiah and Reddy, 1993, Raz and Fluhr, 1992, Trewavas and Knight, 1994). CDPKs and other calcium-binding proteins have been implicated as important mediators in calcium signalling (Roberts, 1993, Roberts and Harmon, 1992).

Since a CDPK was first purified to homogeneity and cloned from soybean (Harmon et al., 1987, Harper et al., 1991, Putnam-Evans et al., 1990), a number of CDPK genes has been cloned from plants, including carrot (Suen and Choi, 1991), *Arabidopsis* (Hong et al., 1996, Hrabak et al., 1996, Urao et al., 1994), rice (Breviario et al., 1995, Kawasaki et al., 1993), maize (Estruch et al., 1994, Takezawa et al., 1996) and mungbean (Botella et al., 1996). Biochemical studies have shown that CDPKs are present in other plants such as zucchini (Verhey et al., 1993), peanut (Dasgupta, 1994), and winged bean (Saha and Singh, 1995).

Calcium-dependent phosphorylation in response to many stimuli has been observed in plants. Examples are touch, and red-light induced protein

phosphorylation (Fallon et al., 1993, Piotrowski et al., 1996), and phosphorylation during lysine catabolism (Karchi et al., 1995). In addition, a substantial amount of evidence that implicates CDPKs in diverse pathways has accumulated in recent years. The large number of isoforms and involvement in diverse pathways suggest that CDPKs participate in calcium-dependent signal transductions as multifunctional enzymes with different specificities. Most studies, however, have been concerned with the observation or suggestion of the involvement of CDPK in the processes and have failed to address direct physiological roles of CDPK in the signalling.

The identification of the interacting proteins has been an important step in dissecting the molecular mechanism of a biological process. There have been several methods developed to that end, such as a blot overlay, a band-shifting assay (Carr and Scott, 1992), and the yeast two-hybrid system (Bartel et al., 1993, Fields and Song, 1989). These techniques have been successful for isolation of novel interacting partners for a number of proteins of interest. Interaction (or expression) cloning is an extension of the blot overlay assay (Blanar and Rutter, 1992, Hirsch et al., 1989). This strategy, which has been used in the identification of substrates and cellular receptors for the protein kinase C (Chapline et al., 1996, Chapline et al., 1993, Jaken, 1996, Ron et al., 1994) and for the cAMP-dependent protein kinase (PKA) (Coghlan et al., 1993, Coghlan et al., 1994, Hirsch et al., 1989), uses a radiolabelled protein as a probe to screen cDNA expression library. It has been noted that the interacting proteins of protein kinases isolated by these techniques are often found to be as substrates as well (Chapline et al., 1993).

The purpose of the present study was to gain more insight into physiological roles of CDPKs in calcium-dependent signal transduction pathways in plants. Questions included what the substrates are, what proteins

they associate with, what physiological processes they control, and what the specificities of the isoforms are. To address these questions, interaction cloning was employed. Three CDPK isoforms, α , β and γ , from soybean were used as probes to screen cDNA expression library made from soybean suspension cell culture to identify proteins that are substrates of CDPKs in vivo, and to determine if there are proteins that target CDPK isoforms to specific locations. Twelve different clones were identified, which interacted only with CDPK γ during screening. These interacting proteins were sequenced, and characterized.

Experimental Procedures

Materials

A complementary DNA (cDNA) expression library, Uni-ZAP™ XR, made from soybean suspension cell culture was a gift from Drs. R. Tenhalan and C. Lamb (Salk Institute for Biological Studies). Plasmids, pET3c/(Ubiquitin)₁ encoding His₆-(Ubiquitin)₁ and p8190 encoding (Ubiquitin)₆, and polyclonal antiubiquitin antibody were gifts from Dr. J. Callis (UC Davis). Bovine ubiquitin was from Sigma. *E. coli* strains, XL1-Blue MRF' and SOLR™, and F1 helper phage, ExAssist™, were from Stratagene. Nitrocellulose filter, Hibond-C extra, was from Amersham. Maltose agarose, glutathione agarose, and iminodiacetic acid agarose were purchased from Sigma.

Interaction Cloning

Interaction cloning was performed with a cDNA expression library made from soybean cell suspension culture and ³²P-autophosphorylated,

recombinant GST-CDPKs as probes. The procedure was a modification of the ones described by Blanar, M.A. and Rutter, W.J. (1992) and Stone et al. (1994). Basic methods for manipulation of phage were based on those described by Ausubel et al. (1995). One milliliter of an overnight culture of *E. coli*, XL1-Blue MRF⁺ grown in LB containing 0.2% (w/v) maltose and 10 mM MgSO₄, was mixed with diluted phage (150,000 pfu) and incubated for 15 min at room temperature. Meanwhile, the top agarose (10 g Tryptone, 5 g yeast extract, 10 g NaCl, and 0.8% agarose per liter) was melted and equilibrated to 50 °C in a water bath. The square culture plates (24.5 x 24.5 cm; Nunc) containing 250 ml LB agar each were prepared as follows. The plates were sterilized by soaking in 75% EtOH and dried prior to use. The agar-filled plates were wrapped with PVC wrap and stored at 4 °C at least overnight before use. The plates were preincubated at 37 °C overnight to reduce the moisture content of the plate. The mixture of phage and cells were transferred to a 125 ml flask containing 30 ml of top agarose and poured evenly on the LB agar in the Nunc plate. After the agar had solidified at room temp, the plate was incubated for 3 - 4 h at 42 °C until small plaques were visible. Nitrocellulose filters which were soaked in 10 mM IPTG in H₂O and air-dried, were overlaid on the agarose and the plate was further incubated for 4-8 h at 37 °C. At the end of incubation, the filter was marked with waterproof ink for the orientation, lifted, and air-dried. The filter was washed in TTBS (20 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) three times for 10 min at room temperature and blocked in a buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 0.5 mM DTT, 5 mM MgCl₂ and 5% (w/v) nonfat dry milk) for 1-2 h at 4 °C.

The probes were prepared as follows. Autophosphorylation of CDPKs was performed in 100 µl reaction buffer (50 mM Hepes, pH7.5, 10 mM MgCl₂, 1

mM EGTA, 1.2 mM CaCl_2 , 0.1 mg/ml BSA, 2 mM DTT) for 15 min at room temperature. The amount of GST-CDPK α , - β , and - γ was adjusted to give 2.5×10^5 cpm/ml (typically 10-30 μg per reaction). The reaction was preincubated for 6 min and initiated by adding 5 μl of [γ - ^{32}P]ATP (10 mCi/mL, 6000 Ci/mmol from DuPont). After 15 min, the reaction was stopped by adding 20 μl of 50 mM Hepes, pH 7.2, 90 mM EDTA, and 5 mM EGTA. The ^{32}P -labeled GST-CDPKs were separated from unincorporated [γ - ^{32}P]ATP by gel filtration (Bio-Gel P-6 gel from Bio-Rad) equilibrated in hybridization buffer (25 mM Hepes, pH 7.5, 10 mM MgCl_2 , 1 mM EGTA, 1.2 mM CaCl_2 , 2 mM DTT, and 50 mM NaCl).

Hybridization was carried out in the hybridization buffer supplemented with 1% nonfat dry milk at 4 °C overnight with continuous, gentle shaking. The initial screening was done with the mixture of the ^{32}P -labeled GST-CDPKs as probes, but later screenings were done with individual GST-CDPK probes. After hybridization, the filter was washed 3 times for 10 min each at room temperature in the same buffer and exposed to the X-ray film.

In Vivo Excision of pBluescript Phagemid from the Lamda Phage Vector Uni-ZAP and DNA Sequencing

A protocol from Stratagene was followed with modification. XL1-Blue MRF' and SOLR cells were grown in LB medium overnight at 37 °C. In a sterile glass tube, 200 μl of XL1-Blue MRF', 200 μl of phage stock (1×10^8 pfu/ml), and 1 μl of ExAssist helper phage (1×10^{11} pfu/ml) were combined and incubated at 37 °C for 15 min. After incubation, 2 ml of LB medium was added and further incubated at 37 °C for 2.5 h. Cells were removed by centrifugation for 15 min at $2,000 \times g$. The supernatant was removed, heated at 70 °C for 15 min and centrifuged for 15 min at $4,000 \times g$. The cleared

supernatant, which contained excised phagemids, was subsequently used to transform SOLA cells. SOLA cells grown overnight, 200 µl, were mixed with 100 µl of excised phagemid stock and incubated at 37 °C for 15 min. After incubation, 50 µl was plated on a LB agar plate containing ampicillin and incubated overnight at 37 °C.

After excision, plasmid DNAs containing the cDNA inserts were purified and sequenced. Sequencing was performed with Sequenase (Amersham) according to the manufacturer's protocol or by Sequencing Core Laboratory, Interdisciplinary Center for Biotechnology Research, University of Florida.

Computer Analysis of Nucleotide and Protein Sequences

DNA sequence analysis was aided by DNASTar (Madison, Wisconsin) for the Macintosh. The nucleic acid and protein data base searches were conducted on the BLAST (Altschul et al., 1990) Network Service of the National Center for Biotechnology Information. Sequence identity was determined by the GAP software program provided by Genetics Computer Group (GCG) thru Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida.

PCR Cloning of 5' End of Clone 16

Primers were designed from 5' side of Clone 16: AS86 (459AGAGTTAGGCAATGCAG₄₄₃), AS88 (309GCTATAACCAAGCTCAAG₂₉₂), and AS85 (124AGGACAAGTCAGTGCATG₁₀₇). The numbers in parentheses were from the sequence of Clone 16. A sense primer, M13R, which was on the 5' side of cDNA insert in the phage vector, was the same for each PCR. The first PCR was done with AS86 as antisense primer and soybean cDNA

expression library as templates. The amplified DNA was size-fractionated on 1.2% agarose gel. A gel piece containing DNA fragments ranging approximately 550 - 1350 base pairs was excised, and the DNA was eluted and used as a template for the second PCR. The antisense primer for the second PCR was AS88. The PCR product was cleaved with *Xba*I and *Kpn*I, and subcloned into *Xba*I and *Kpn*I digested pUC19. The presence of an insert with 5' sequence of clone 16 was confirmed by colony PCR with 3' primer, AS85. The clone with the longest insert based on 2% agarose gel electrophoresis was subsequently sequenced.

Construction of Plasmids

PCR was performed for all clones. Except for clone 3, PCR conditions were as follows: 1 cycle at 94 °C for 5 min, 20 cycles at 55 °C for 1.5 min, 72 °C for 1.5 min, and 94 °C for 1 min and last cycle at 55 °C for 1.5 min and 72 °C for 10 min. Because of low GC contents in the primer, the PCR condition for clone 3 was same as above except that the annealing temperature was 48 °C.

pMal-cRI/GIP3

The sense primer was GCGGATCCCATATGCCACTTGTGTT (*Bam*HI, *Nde*I) and the antisense primer GCGGATCCCTATATAACATAATC (*Bam*HI). The template for PCR was Clone 3. *Bam*HI digested fragment was ligated into the *Bam*HI site of pMal-cRI. The integrity of subcloning was verified by complementation assay described elsewhere.

pMal-cRI/GIP6

The sense primer was GCGGATCCCATATGGGTAAGGGAACG (*Bam*HI, *Nde*I) and the antisense primer GCGGATCCTTAGGTAGAGGCAGC (*Bam*HI) and template was Clone 6. The product was digested with *Bam*HI and inserted into pMal-cRI.

pETH-3b/GIP8 and pET-15b/GIP8

The sense primer was GCGGATCCCATATGTCGGACGAGGTA (*Bam*HI, *Nde*I) and the antisense primer GCGGATCCCTATTTCTGA TTCAAGTG (*Bam*HI) and Clone 8 as a template. The PCR product was cleaved with *Bam*HI and the insert was ligated into pUC19Δ*Pst*I whose *Pst*I site was deleted by cutting, filling-in, and religation. The region between *Nhe*I and *Pst*I sites was replaced with the same region of the original clone to minimize PCR errors. The resulting plasmid contained three *Nde*I sites (two were internal and one was on 3' side of the vector). Since digestion with *Nde*I and *Bam*HI could yield three fragments (*Nde*I - *Nde*I, *Nde*I - *Bam*HI and *Bam*HI - *Nde*I) and the latter two were very similar in size, *Bam*HI digestion was carried out first to isolate *Bam*HI-*Bam*HI fragment and the fragment was further digested with *Nde*I. The resulting *Nde*I - *Nde*I and *Nde*I - *Bam*HI fragments were ligated first, and the ligates were used in a second ligation reaction with pETH-3b digested with *Nde*I and *Bgl*II or with pET-15b digested with *Nde*I and *Bam*HI. The orientation of the insert was verified by restriction enzyme digestion.

pMal-cRI/GIP12

The sense primer was GCGGATCCCATATGTTAGGTGTTCTGCT (*Bam*HI, *Nde*I) and the antisense primer GCGGATCCAAGCTTTAG ATCCAAG (*Bam*HI) and Clone 12 as a template. The *Bam*HI digested fragment was inserted into pUC19Δ*Hind*III. The swapping was done at the *Hind*III-*Hind*III region. The plasmid was digested with *Bam*HI and the *Bam*HI-*Bam*HI fragment was isolated and inserted into pMal-cRI.

pMal-cRI/GIP14 and pET-15b/GIP14

The insert encoding GIP14 was amplified by PCR using the sense primer GCGGATCCCATATGTCATCAAAGCTTC (*Bam*HI, *Nde*I) and the

antisense primer GCGGATCCTCAGCAGAACGAGAG (*Bam*HI) and Clone 14 as a template. The PCR product was digested with *Nde*I and *Bam*HI and ligated into pUC19 Δ *Hind*III whose *Hind*III site was deleted by digestion, filling-in, and religation. *Hind*III - *Stu*I fragment of the resulting plasmid was swapped with *Hind*III and *Stu*I fragment from original Clone 14. The resulting plasmid was digested with *Nde*I and *Bam*HI and the fragment was inserted into pET-15b, or filled with klenow and ligated into pMal-cRI treated with *Bam*HI and klenow. The orientation of the insert was verified by restriction enzyme digestion.

pMal-cRI/GIP16KD

The insert encoding the kinase domain of GIP16 was PCR-amplified using the sense primer, GCGGATCCAAGAAGAGGGAGGGGAGT (*Bam*HI) and the antisense primer, GCGGATCCTCAAAATGGGGTAACAC (*Bam*HI) and clone 16 as a template. The insert was cut with *Bam*HI and subcloned into pUC19 Δ *Hind*III. *Hind*III-*Sty*I fragment was swapped with the original fragment from Clone 16. The resulting plasmid was digested with *Bam*HI and *Bam*HI-*Bam*HI fragment was inserted into pMal-cRI.

Expression and Purification of Recombinant Proteins

Bacterial hosts for expression of recombinant proteins were PR745 for pMal-cRI vector or BL21(DE3)pLysS for pET vectors. The expression conditions were as described previously except that MBP/GIP16KD was expressed at room temperature with slow shaking (100 rpm). For purifying MBP fusion proteins, cells were resuspended in the resuspension buffer (50 mM Tris, pH 7.5, 150 mM KCl, 10 mM EDTA, and proteinase inhibitors). Cells were sonicated and centrifuged. Cleared lysates were loaded on a maltose agarose column (generally 1 - 5 ml packed volume) which was equilibrated

with Buffer A (50 mM Tris, pH 7.5, and 150 mM KCl). The column was extensively washed with Buffer A and proteins were eluted with Buffer A supplemented with 10 mM maltose.

GIP8 which was in native form was purified by using glutathione agarose column equilibrated with Buffer A and eluted with Buffer A supplemented with 10 mM reduced glutathione. His₆/GIP14 was purified with iminodiacetic acid column as described previously. The purified proteins were concentrated with a Centricon 10 or 30 (Amicon) and the buffer composition was adjusted to have 2 mM DTT, 40% glycerol, and 10% ethylene glycol. The proteins were stored at -20 °C or at 4 °C.

Phosphorylation Assay

Interacting proteins were phosphorylated with [γ -³²P]ATP (500 cpm/pmol) in the kinase assay buffer described previously. The reaction was carried out for 15 - 30 min at room temperature (20 - 25 °C) with affinity-purified recombinant proteins or with cell lysates. The reaction was stopped by adding 2x gel loading buffer (0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol). Phosphoproteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and the gel was autoradiographed. When the effects of GIPs on CDPK activity were assayed, Syntide-2 was used as a substrate and the reaction was performed as described previously.

Interaction of GIPs with CDPKs

Purified GIPs or cell lysates containing expressed recombinant GIPs were incubated with either GST/CDPKs - agarose (10 μ g) or GST-agarose (10 μ g) for 30 - 60 min at 4 °C. The buffer composition was the same as in the

protein kinase activity assays described in Chapter 2. The agarose was pelleted and washed three times with the buffer. Proteins were eluted with 10 mM glutathione, 50 mM Tris, pH 7.5, 150 mM NaCl, and 2 mM DTT and resolved by SDS-PAGE. Proteins were transferred onto nitrocellulose and immunoblotted with anti-maltose binding protein (MBP) antibody or with anti-ubiquitin antibody. When maltose agarose was used, the preloaded protein was MBP-fusion GIPs and immunoblotting was developed with antibody raised against soybean CDPK or with polyclonal anti-CLD antibody.

Glutathione S-Transferase(GST) Activity Assay

GST activity was measured as described in Mannervik and Guthenberg (1981) with modification. To a 1 ml cuvette were added 500 μ l of 0.2 M Tris (pH 7.2), 50 μ l of 20 mM GSH, a suitable amount of enzyme and deionized water to final volume of 1 ml. The reaction at 25 °C was initiated by addition of 50 μ l of 20 mM 1-chloro-2,4-dinitrobenzene (CDNB). The reaction was monitored spectrophotometrically by the increase in absorbance at 340 nm. When the effect of phosphorylated CDPK was analyzed, the buffer composition was the same as in the kinase assay buffer described in Chapter 2.

In Vitro Translation

In vitro translation assay was carried out as described in Cline et al., (Cline et al., 1985). Pretreatment of the wheat germ extracts was with either 100 ng of CDPKs and 0.5 mM CaCl_2 , 0.5 mM CaCl_2 alone, 100 ng of GST-CDPK α (1-312), or 2 μ M of staurosporin with or without 0.5 mM CaCl_2 for 15 min at room temperature and used in the assay. The control experiment was performed with either untreated extracts or pretreated ones with the enzyme storage buffers described above.

Results

Interaction Cloning

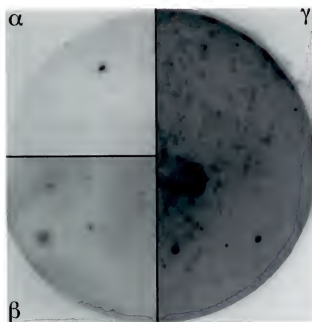
To identify proteins that interact with soybean CDPKs, interaction cloning (Blancar and Rutter, 1992, Stone et al., 1994) was used. A complementary DNA (cDNA) expression library in Uni-ZAP™ XR vector made from suspension-cultured soybean cells was screened with probes that were a mixture of recombinant GST-CDPK α , β , and γ labeled with ^{32}P by autophosphorylation. In an initial library screening, 150,000 pfu were plated and screened. Thirty-five positive plaques were picked and 16 plaques were randomly selected for further purification. Out of 16 positive clones, four did not interact with the mixture of CDPKs in the subsequent screenings. The twelve positive plaques were purified for subcloning. Two purified plaques (Clone 4 and 13), however, were not further examined because of the difficulties in DNA isolation (Clone 13) and a short insert size (Clone 4).

Purified Plaques Interact Only with CDPK γ

The specificity of interaction of purified phage was determined with individual GST-CDPKs labeled with ^{32}P as a probe for hybridization (Fig 3-1). All plaques interacted only with GST-CDPK γ . Longer exposure of the filters to the X-ray film did not reveal any interaction of CDPK α or β with the proteins encoded by the cDNAs (data not shown). The cDNA inserts were designated Gips for CDPK γ (gamma) interacting proteins and the proteins encoded by the Gips were named GIPs.

Figure 3-1. Purified clones encode peptides that interact only with CDPK γ .

Three plates were prepared from the same phage stock to test the specificity of interaction. The purified recombinant phage were induced to express protein, and blotted onto nitrocellulose membranes. Each membrane was incubated separately with ^{32}P labelled GST-CDPK α , $-\beta$, and $-\gamma$. Shown here is the phage containing Clone 8. Experiments with other clones showed the same specific interaction only with CDPK γ (data not shown).



Sequencing and Characterization of cDNA Clones

The purified phage was subjected to *in vivo* excision to release the phagemid vectors, pBluescript, bearing the cDNA inserts at *EcoRI* and *XhoI* sites. The cDNA inserts in the excised phagemid vectors were sequenced. Table 3-1 summarizes the sizes of cDNA inserts and of proteins encoded by the Gips and Figure 3-2 represents nucleotide sequences of the Gip cDNA clones and the deduced protein sequences.

A homology search of the sequences of Gips against nucleic acid and protein databases identified 8 out of 10 Gips (Table 3-2). Gip1 (Fig 3-2A) has an open reading frame that is 100% identical (at the nucleic acid and protein level) to the polyubiquitin gene from soybean. It contains a complete open reading frame that encodes four tandem repeats of the ubiquitin coding unit, which is a characteristic of genes encoding ubiquitin (Belknap and Garbarino, 1996, Gonda, 1994).

The protein sequence of Gip8 (Fig. 3-2F) is also 100% identical to a known protein from soybean but the DNA sequence of Gip8 has 4 mismatches: one in the open reading frame but at the wobble position; the other three in the 3' untranslated region. The protein in the database is reported as glyoxalase I since the partial peptide sequences used to identify the cDNA is derived from the protein purified by following the glyoxalase activity (Paulus et al., 1993). The sequence, however, was 64% homologous to a tobacco glutathione S-transferase (GST) (Van der Zaal et al., 1991) at the protein level, and had no apparant homology to a tomato glyoxalase I (Espartero et al., 1995). Therefore, the protein encoded by Gip8 was designated soybean GST (sGST) (see Discussion). The DNA sequence of Gip8 also

Table 3-1. Characteristics of cDNA clones

Clone	Length ^a		MW ^b	Clone	Length ^a		MW ^b
	bp	aa			bp	aa	
Gip1	1,158	305	34,195	Gip8	955	219	25,903
Gip2	510	92	10,082	Gip12	1,040	229	24,747
Gip3	1,341	342	37,046	Gip14	1,062	161	17,581
Gip5	424	65	7,335	Gip15	1,184	148	16,147
Gip6	496	95	10,722	Gip16	2,144	635	68,575

^aLength in aa, and predicted molecular weight (MW^b) are given from the longest reading frame or from the putative opening reading frame. bp is base pair and aa is amino acid.

Table 3-2. cDNA clones encoding proteins that show sequence identities to known genes.

Clone	Description ^a	Length (aa) ^b	Identity, % ^c
Gip1	Ubiquitin (Soybean, D28123)	305	100
Gip3	Serine acetyltransferase (Arabidopsis, L34076)	342	71
Gip6	Ribosomal protein L37 (Arabidopsis, F20017)	95	82
Gip8	Glutathione S-transferase (Soybean, P46417)	219	100
Gip12	Cytochrome b561 (Human, U06715)	229	38
Gip14	Calmodulin (Plasmodium, X56950)	161	40
Gip15	Translocase of outer mitochondrial membrane (Potato, X92491)	148	76
Gip16	Transmembrane kinase-like protein (Arabidopsis, P33543)	702	74

^aA description of the best data match is given together with database accession number and the source of the match in parentheses.

^bLength in amino acids (aa) compared is from putative open reading frame or longest reading frame of each clone.

^cPercentage represents amino acid identity of the length compared.

A. *Gip1*

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1  TCTCTGTGATTTTCCCTACCTCTCCCTTCAAGATGCAGATCTTCGTCAAGACCCCTCACCG 60
   S V Y F P T S P F K M Q I F V K T L T G
61  GCAAGACCATCACCTTGGGTGAAAAGCTCTGACCATCGACAACGCTCAAGGCCAAGA 120
   K T I T L E V E S S D T I D N V K A K I
121  TCCAGGACAAGGAAGATCCCCCGGACCAGCAACGCTCTCATTTTCGCCGAAAGCAAC 180
   Q D K E G I P P D Q Q R L I F A G K Q Q L
181  TTGAGGACGGCGTACCTTGTCTGACTACAACATTAGAAGGAGAGTACTCTTCACTCG 240
   E D G R T L A D Y N I Q K E S T L H L V
241  TCTCCGTCTCCGTGGCATGACAGATCTTCGTTAAGACACTCACCGGCAAGCATAA 300
   L R L R G G M Q I F V K T L T G K T I T
301  CCCTAGAGGTTGAAAGCTCCGACACCATCGATAACGTCAAGGCCAAGATCCAGGACAAG 360
   L E V E S S D T I D N V K A K I Q D K E
361  AGGGTATCCCCCGGACCAGCAACGCTCTCATCTTCGCCGAAAGCAGCTCGAGGACGGCC 420
   G I P P D Q Q R L I F A G K Q L E D G R
421  GCACCTCGCCGACTACAACATCCAGAAGGAATCAACCTTCACTCGTCTCCGTCTCC 480
   T L A D Y N I Q K E S T L H L V L R L R
481  GTGGTGGCATGACATCTTCGTTAAGACCTCACCGCAAGACTATTACCTAGAAGTCG 540
   G G M Q I F V K T L T G K T I T L E V E
541  AAAGCTCCGACACCATCGACAACGCTCAAGGCTAAGATTAGGACAAGGAGGAATCCCC 600
   S S D T I D N V K A K I Q D K E G I P P
601  CAGACCTCAGAGGCTGATCTTCGCCGGAAGCAGCTCGAGGACGGACGCCCTTGTCTG 660
   D Q Q R L I F A G K Q L E D G R T L A D
661  ACTACAACATCCAGAAGGAGTCAACTCTCCACTTGGTGTTCGTCCTTCGTTGGTATGC 720
   Y N I Q K E S T L H L V L R L R G G M Q
721  AGATTTCGTGAAGACTCTTACGGGTAAGACTATTACCCTCGAGGTGGAGAGCTCTGACA 780
   I F V K T L T G K T I T L E V E S S D T
781  CCATTGACAATGTGAAGGCCAAGATTAGGACAAGGAATCCACCGGACCCAGCAGA 840
   I D N V K A K I Q D K E G I P P D Q Q R
841  GGCATGATTTTGTCTGCAAGCAGCTCGAGGATGGAAGGACCCCTCGCTGACTACAACATCC 900
   L I F A G K Q L E D G R T L A D Y N I Q
901  AGAAGGAATCAACCTTCACTTGTCTCCGTCTCCGTGGGGGGTTTAAAGCTCGTTGTG 960
   K E S T L H L V L R L R G G F *
961  TAATGTTGGATGTGTCCCAAAACATTGAAGAACTTTGATGTTTAAATGGGTCTGTAATA 1020
1021  ATGTCCTTGAATAAAGTTCGGTTTGTGTTGAATCAATTGTGCCCATTAATTAAGT 1080
1081  ACTCTAATATCCCACTGTTTGTATGAATGTGGAATATGAATGATTAAATGTCA 1140
1141  AAAAAAAAAAAAAAAAAA 1158

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B. *Gip2*

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1  TAATATCTCCGGCGTCAAGACCGTCACCTTCCCCGAGTTGACTTTTAAGTTCAAAGGCGG 60
   N I S G V K T V T F P E L T F K F K G G
61  CGCCAAAATGACGACGCCGCTTCAGAATTATTTCTCATTGGTCCGGAGATGCCGAGGTCGT 120
   A K M T Q P L Q N Y F S L V G D A E V V
121  GTGTTTACCGTTGTCTCCGACGAGGTGCCGCTCCGCCAAGACAACCGGTCCGGCCAT 180
   C L T V V S D G G A G P P K T T G P A I
181  CATCTTGGGGAATTATCAGCAGCAGAATTTCTACATTGAATATGATTGGGAATGAGAG 240
   I L G N Y Q Q N F Y I E Y D L E N E R
241  GTTCGGATTGGTCTCGTAGTTGCAGAAAGGAAGCTTAGTTTATAGTTGAAATAATTA 300
   F G F G P R S C R R K A *
301  TTAACACCAATTTCGATTACATGACATCCACGACGTTGTGGTGGTTATAGTCTATTATT 360
361  ATTATTATGATTATTGTGCTTCTATTGATTTTATCTTATTTTTTACTGCGTAAG 420
421  TGCAGGAACAGAGTATGGATGCTTAGAATGATTTTAAATAAATTGAGATATACTATATGA 480
481  TTAGTTCAAAAAAAAAAAAAAAAAA 510

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Figure 3-2. Nucleotide sequence of the *Gip* cDNA clones and the deduced protein sequence.

Nucleotide sequences of the *Gip* cDNAs are shown in the numbered rows and the predicted amino acid sequences (single letter code) of the longest open reading frames are shown beneath the corresponding cDNA sequence.

C. Gip3

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1  ATTTTCTTCATCCCCAAAGTTTCAATCTTTGTATTCAATTCACCCCTTTCTCTCTCTCC 60
61  ATGGCCACTTGTGTTGATGCCCAACCCCTCTTTCTCGGAAGCCTAACGGGTGCCAATCC 120
    M A T C V D A P T P L S R K P N G S Q S
121  GATGATCGTTCTCTCAATTACATGAATTCCTGCCGACCACCTTTCTCTGATCGGTTCC 180
    D D R S F N Y M K F C R P T F S D R V P
181  TGCATACCCATTTCGAAGAATCGTGACATGCCTTCACACGCGTGGAGGAGTTTGACACA 240
    C I P I C K N R D T A F T R V E E F D T
241  TGTGTGGAGGATAATGATGATATTGAGGTTGAGGTTGTGGAGGTTGTTGATCTTTGGTTG 300
    C V E D N D D I E V E V V E G V D L W L
301  AAGATTCAAGAGGAGGCAAGGTTAGATGTTGATCAAGAACCAATTTTATCCAGCTACTAT 360
    K I Q C E E A R L D V D Q E P I L S S Y Y
361  TTCAGTTCCATTTTGTCTCACAAATCTTTGGAGAGTGCCTTGCCCAACCCCTCTCCACA 420
    F S S I L S H K S L E S A L A N H L S T
421  AATTTGAGCAGCTTGAGCCTTCCAAGCAGCACCCCTTTTGAGCTTTTTCATGGGTGTTTG 480
    N L S S L S L P S S T L F E L F M G V L
481  GTTGATGATGGAGATGATGACATCGTAGGTGCTGTGAAGGATGATTGATAGCTGTTAAG 540
    V D D G D D D I V G A V K D D L I A V K
541  GAGAGGGACCCCTGCTTGACATAAGTTATGTGCATTGCTTGCTGAATTTCAAGGGTTTTTG 600
    E R D P A C I S Y V H C L L N F K G F L
601  GCATGTCCAGGCTATAGGATTGCTCACAAATTTGGCTTCAAGGAGGAAGTCTTGCGG 660
    A C Q A H R I A H K L W L Q G R K V L A
661  CTGTTGATTGAGAATAGGGTGTCTGAGGTTTTTGTGTGGATATTCACCTGTGGTCCAAA 720
    L L I Q N R V S E V F A V D I H P G A K
721  ATTGGACGTGGGATTTTGTGATCATGCAACAGGACTTGTGTGGGGAGACTGTCAGTT 780
    I G R G I L L D H A T G L V V G E T A V
781  ATTGGGAATAATGTGTCAATTTTGCATAATGTGACATTGGGAGGCACTGGTAAGGCAAGT 840
    I G N N V S I L H N V T L G G T G K A S
841  GGGGATAGACCCCTAAGATTGGTGATGGGGTGTGTAGGTGCAGGGACTGTGATTTTG 900
    G D R H P K I G D G V L I G A G T C I L
901  GGGAAACATTAAAGTTGGTGATGGAGCTAAGATTGGTGCTTGTCTGTTGTTGAAGGAA 960
    G N I K I G D G A K I G A C S V V L K E
961  GTGCCACCAAGGACTACTGCTGTTGGGAACCCCTGCTAGGTTGGTTGGAGGGAAGATAAC 1020
    V P P R T T A V G N P A R L V G G K D N
1021  CCTATTAAATTGGATAAGATGCCTAGTTTACCATTGGACCATACTTCTATGCTGATTAT 1080
    P I K L D K M P S F T M D H T S W S D Y
1081  GTTATATAGAAGCTAATTAATTGCTAACATGTTTTAGTGTTTGGTGGGGAATT 1140
    V I *
1141  GTTTTGGTTGAGGGGCTTGGTTGTTGTGCAAGAGAATCTAAGTTCTCCTGCTGACAA 1200
1201  CAGGGCGTCCCTTGAATCATCGTGTAGATTTTAAAGAATAGTTAGATGTAGTACTTTG 1260
1261  TTGTTGAAGGGGCCATGATGACAACCCCTTTGTGTAAAATTTATGAATATGATATTCA 1320
1321  GCTTGTATTGGTTAAAAAAA 1341

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D. Gip5

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1  AACAAAGATGAAAGGTTTGCAAAAAGGGTGTCCTCAATACATAGCCAGGTTATGAAGATC 60
    N K D E R F A Q K G V P I H S Q V M K I
61  AGGCAAGAGTCAGAGAAAATTGTTGATTGGTCCCGGGCAAGCCAGAGATAAGACCCGTT 120
    R Q G E S E K K I V D W S P G K P E I R P V
121  CTCCTGAAATAAGCCGCGAGATTTCGCCGTGCGCGTTGGGGATCTCCGGCAGACCGATT 180
    L R E I S R Q I S R S P L G I S G R P I
181  TCCGTTGGTGACTCATAGGTTAAGAACATGGTTAAGGGTTTTGGATTAGTGTTGTATGT 240
    S V G E S *
241  ACACAGGTGGAAGAGGGAGGATTTGTGTGCTTCCCCTGCTCCTGTTCTGTTTCTCTCTGT 300
301  TTTTGTGTCAGAGAAATTTAGGCATATTTAGCTATTTTGTCTCCACTTTTGTGTAATAAT 360
361  AATAATAGTGTATTGGATATAATATATCATGTGTTTTTAAAAAATAAAAAAAAAAAAAA 420
421  AAAA 424

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Figure 3-2 continued

E. Gip6

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1  ATTAGGGTTTTTTGTGATCTGCAAGCTACGATGGGTAAGGGAACGGGAGTTTCGGTAAGA 60
      M G K G T G S F G K R
61  GAAGGAACAAGACCCACACCCCTCTGTGTGAGGTGTGGCCGCCGAGTTTCCACCTCCAGA 120
      R N K T H T L C V R C G R R S F H L Q K
121  AGAGTCGTTGGCCGCGTGCCTTTCCCGCTGCGCGCACCAGGAAATATAACTGGAAGCG 180
      S R C A A C A F P A A R T R K Y N W S V
181  TGAAGGCCATTAGGAGAAAGACCACCGAACTGGAAGGATGAGTACTTCGCTAACGTGC 240
      K A I R R K T T G T G R M R Y L R N V P
241  CCCGTAGATTTAAGAGTGGCTTCAGAGACGGTACCGAAGCTGCACCCAGGAAGAGAGAG 300
      R R F K S G F R D G T E A A P R K R G A
301  CTGCTGCCTCTACCTAAGCTTTGAATAATAGCAATTTTGTCTGGTCACTGAATTATATA 360
      A A S T *
361  GGATTTAATGTTTCTATGTGTATGCCGAGCTCTTTGGAACACGTTTCTTCTGTGCGTTTT 420
421  AATTTTCATTTAATATGTAGTGGCTTTGTATTTTCGACGTGAGGATTGCAAGGAATTTTGT 480
481  TTTTCAAAAAAAAAA 496

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F. Gip8

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1  CAAACTTGAGTTGGATTTTGGTGATCTTGTGAATTGATCTGCGATGTGCGACGAGGTAGT 60
      M S D E V V
61  TCTGTTGGATACATGGGCTAGCATGTATGGTATGAGGGCTAGGATTGCATTGGCTGAAAA 120
      L L D T W A S M Y G M R A R I A L A E K
121  GGGTGTTAGGTATGAATACAAGGAAGAGAATCTCATGAACAGGAGTCCCTTTGCTTTTGA 180
      G V R Y E Y K E E N L M N R S P L L L Q
181  GATGAACCAATTCACAAGAAGATTCTGTGTTGATCCATAATGGCAAAACCCATTGTGA 240
      M N P I H K K I P V L I H N G K P I C E
241  ATCTGCAATAATAGTGCAGTACATTGATGAGGTCTGGAATGACAAGTCTCCACTCATGCC 300
      S A I I V Q Y I D E V W N D K S P L M P
301  CTCGATCCCTTACAAGAGATCTCAAGCCAGATTCTGGGTGGACTACATTGACAAAAAGAT 360
      S D P Y K R S Q A R F W V D Y I D K K I
361  TTATGATACTTGGAAAGAAATGTGGCTTTCTAAAGGAGAAGAGCATGAAGAAGGGAAGAA 420
      Y D T W K K M W L S K G E E H E E G K K
421  GGAGTTGATCTCTATCTTTAAGCAATTAGAAGAGACACTAACTGACAAAACCCCTTTTATGG 480
      E L I S I F K Q L E E T L T D K P F Y G
481  GGATGACACGTTTGGCTTTGTTGATCTTGTGATCATTCTCTAGTTGGTTTTATAC 540
      D D T F G F V D L C L I T F S S W F Y T
541  TTATGAGACATATGGGAACCTCAAAATGGAAGAAGAGTGTCTAACTCATGGCTTGGGT 600
      Y E T Y G N F K M E E E C P K L M A W V
601  CAAGATCGATGGAGAGAGAGACTGTGTCCAATACTCTCTGATGCTAAGAAGGTGTA 660
      K R C M E R E T V S N T L P D A K K V Y
661  TGGTCTTATTGTGGAAGTGCAGAAGACACTTGAATCGAAATAGAAGATTTCAATAAATCA 720
      G L I V E L Q K T L E S K *
721  ACCCATTAATAATATTTTCATGTTAAATATGTTGTTGAAGGTCTTGTGTACTTTTCCT 780
781  CTATGGTGTGTGGGTGGGTGAGTCACTTATGTGGTTTACTAGACACTAATACATCTTC 840
841  TCTATTGAAGATTAAAGGATCTAATATTTTCCCATTAATATATGAATATTATCGT 900
901  CTTTGACATTGAAAAGAAAGAAAACGTAGAAAAGCAAAAAAAAAAAAAAAAAA 955

```

Figure 3-2 continued

G. Gipl2

1 TTTAGGTGTTCTCGTCTTCCGTTGACATACGTGGCTCACGTTCTGGCAGTTGTTGCCAG 60
 L G V P A L P L T Y V A H V L A V V A S
 61 CGTCTTGGTCTGGTCTGGGTCGTCATTTAGGGGTGGCTTGGCTTGGGATTCTGATAA 120
 V L V V V W V V N F R G G L A W D S D N
 121 CAAGTCCCTCATCTTCAACATTCACGATTCTTATTGTTATTGGCTTACTAGTTTCATGG 180
 K S L I F N I H P V L I V I G L L V H G
 181 GGGTGAAGCTATTATAAGTTACAAAGCTTTTCCCTAAAGAAGGTAAGAATAATGAT 240
 G E A I I S Y K A F P L K K E V K K L I
 241 ACACCTTGTTTCCATGCCATCGCATTAACTCTGGAAATGTTGGAAATTATACTGCCTT 300
 H L V F H A I A L I L G I V G I Y T A F
 301 CAAGTTTCACAATGAAAGTGGGATTGCCAATCTCTACAGCTGCATTCATGGCTTGAAT 360
 K F H N E S G I A N L Y S L H S W L G I
 361 TGGGGTATTGTCCCTTTATGGCATTCACTGGATATATGGGTTTGATGCTTTTTCTTCCC 420
 G V I V L Y G I Q W I Y G F V I F F F P
 421 TGGTGGGACTCCAGACATTAGACGCGCTCACTTCCTTGGCAGCGCTATTGGGCTGT 480
 G G T P D I R R A S L P W H A L F T G L F
 481 TGTATTGTCTTGGCTGTGGGCACTGCTGCTCTGGGTTTCTGGAGAAGCTCACTTCTCT 540
 V F V L A V G T A A L G F L E K L T F L
 541 TGAGAACTCAGGATTGGACAAATATGGTCTGAGGCTTGTCTGTCACTTCATGCCAT 600
 E N S G L D K Y G S E A L L V N F T A I
 601 TATCAAACTCTTATTGGTGCCTTTGTGTGTATCTGCTCTAGCTGATGCTCCCCAGC 660
 I T I L F G A F V V L S A L A D A P A
 661 CCCTGCAGCAGATGACTATGAAGCCATATAGACATCTGAAGTTGTATCATATTTTTAT 720
 P A A D D Y E A I *
 721 TTTATGGAATAATCTCATGTCTGCTATAAAAGTTTGTATTGGGAGTTGGAGGAAGGAAAT 780
 AATTGTGTTGTATTCTTGGACTAAAGCTTGTATTAACTCATCAATTTCAATATATT 840
 841 AACCCATAAATGTGAAATAGTCACTGGGTATATCAGTTTCTTTTGTGTTGAACCT 900
 TTCCTTGATCAGCAGTTGAGATTAGCTCATATAAATTTGTGTGACAGTACATTGAAAATTC 960
 961 AGTGTATCTTGTAATTTTATTCCTTTGTGAAGATATGTACATCATATATTTTCAAAAA 1020
 1021 AAAAAAAAAAAAAAAAAA 1040

H. Gipl4

1 TGCAAAATCCCTGTGCATGTATTAGTTTGTGATTGATTTTTTCTCTGAGGGGGGCACA 60
 61 TTAATTTGTGCTCAATACTCATACATTGCGGTTCCCTTATTATTTTGAATTCATTACATT 120
 121 GATATTGATTAACATGTCAATTTGTCAAAAAGAGTCTAATTTAGTTGGTTGATCAGAGTGT 180
 181 GTGATAGTCTTCGATTCCACGGATTAAAAAGAAAATTAACATGTCAAAAGCTTCAAGT 240
 M S S K L Q V
 241 TCAGCAGCTTAATCAGTTAAGGGAGATTTTCGGTCGATTGACATGGACTCAGATGGAG 300
 Q Q L N Q L R E I F G R F D M D S D G S
 301 CCTAACAAATGCTAGAGCTAGCAGCACTTCTTAGGTCTCTGGGGCTGAAGCCCTCGGGTGA 360
 L T M L E L A A A L L R S L G L K P S G D
 361 CCAAGTCCAAGCACTGTTAGCCAACTAGGACTCAACGCCAATGGCAAGTGGAGTTTGA 420
 Q V Q A L L A N M D S N A N G K V E F D
 421 TGAATTGATAAGGCCATATTACTGACATAAATGCACAGGTTTGTCTGAACCAAGAAC 480
 E L I R A I L P D I N A Q V L L N Q E Q
 481 GTCCTTAGGGGTGTTCAAGTGCTTCGATCGCAGCGCAACGGTATCATATCGGCCGAGA 540
 L L G V F K C F D R D G N G Y I S A A E
 541 GTTGGCCGGGGCAATGGCCAAAATGGGCCAGCCACTCAGTACCGAGAGCTCAGGAGAT 600
 L A G A M A K M G Q P L T Y R E L T E M
 601 GATCAAGAGGCTGACACGGATGGGATGGTGTATTAGCTTCACTGATTTTGCCACTAT 660
 I K E A D T D G D G V I S F T E F A T I
 661 CATGGCTCGCTCTGCTTCTGATTCTTAGGCCTCTCGTTCTGCTGACCGGTATAACATATA 720
 M A R S A S D F L G L S F C *
 721 TTCATTTCACTAAGTTGTACAAATATCTTGATTTTTTCCCTTTTGAATGAATGAATCA 780
 781 ATCAAAATATGATATTTGGTTGTACTGGGTATTACTAATACTAATTACCCCAATTCCT 840
 841 GCCAATTTCACTGCTTTTTTAAAAAAATTTAAATTTGAATTAAGCTAATTAATCAAGGT 900
 901 TTTATATGCTTTGTAAGAAATTCGTAATAAATTTTTCATACCATTTAGAGTGCAATT 960
 961 CTATCCGTGTGTTGAATGGCTACATATTTTCAAGTCAATTACAGAGAAAGATGTAAAA 1020
 1021 TGAAGAGAATAGCTTCTATGTTAAAAA 1062

Figure 3-2 continued

I. Gip15

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1   CTGTTTCGAAGCTAGAGGAGGCGCTTGCAGTGAATCCCAAGAAGCATGACACTCTTTGGT    60
   V S K L E E A L A V N P K K H D T L W C
61  GCCTGGGAAACGCTCACTCTCGCAAGCGTTTTTAATTCGGGACCAGGAGGAGGCAAAAG    120
   L G N A H T S Q A F L I P D Q E E A K V
121 TTTATTTTGATAAGGCAGCTGTGTACTTTCAGCAAGCTGTTGATGAGGATCCTTCAAATG    180
   Y F D K A A V Y F Q Q A G V D E P S N E
181 AACTTATCCGCAAAATCATTGGAATGGCTGCTAAGGCTCCTGAGCTGCATGTGAAATCC    240
   L Y R K S L E V A A K A P E L H V E I H
241 ATAAGCAGGGAATTGTGTCAGCAGCAGCAGGCGGAGCAGCAAGCTGGATCTTCTACTCTG    300
   K Q G F G Q Q Q Q A A A T A G S S T S A
301 CTTCCGGTACAAAGACTCAGAAGAAAAAGAGCAGTGATCTGAAGATGACATATTTG    360
   S G T K T Q K K K K S S D L K Y D I F G
361 GATGGATAAATCTGGCAGTCGGCATTGTTGCATGGGTGGGATTTGCAAAATCAAACCTGC    420
   W I I L A V G I V A W V G F A K S N L P
421 CACCGCCTCCCCCTTCCCCCTCCTCGATAAGTGAAGCCATTGATTGTATCTCACACATTA    480
   P P P P S P P R *
481 TTGATTATTGAAATGATATTTGATTTCGGTTAGTGTCGTTTATTAGTTTGCAGTAGT    540
   AATGTTTGTGCATGGTGAGCCGTGGCTTATCTTATCCATCATACAAAATTTCTTACTG
541 AATGTTTGTGCATGGTGAGCCGTGGCTTATCTTATCCATCATACAAAATTTCTTACTG    600
   TGGGCTGAGCAGTTAAAAATGTCCGCTTAGATGTTGCTAGTATTGCAAAATCAAAG
601 TGGGCTGAGCAGTTAAAAATGTCCGCTTAGATGTTGCTAGTATTGCAAAATCAAAG    660
   CATTGCCAAATTTGCCATCAGTTAAGTATTGAAAAATAAAGAATTTTGCTGCAAAAAA
661 CATTGCCAAATTTGCCATCAGTTAAGTATTGAAAAATAAAGAATTTTGCTGCAAAAAA    720
   AAAAAAATAAAGAATTTTGCTGCAAACTTCAAAGCCAGCTGATGAGGCCAAGGCTTCAAGC
721 AAAAAAATAAAGAATTTTGCTGCAAACTTCAAAGCCAGCTGATGAGGCCAAGGCTTCAAGC    780
   ATGATGGCAGGGGGCTTTCAGTAGTTAATCGAGTTTTCATGTTTCAGTTTGTGCCCTG
781 ATGATGGCAGGGGGCTTTCAGTAGTTAATCGAGTTTTCATGTTTCAGTTTGTGCCCTG    840
   CTTTAATAGGAAGAACCACAAAAAGAAAGTAATAAAGAAGAAAATTTGGATAGCTTT
841 CTTTAATAGGAAGAACCACAAAAAGAAAGTAATAAAGAAGAAAATTTGGATAGCTTT    900
   TAAGATTGGAATTGAAGCTGGTGTGTAATTAAGTAATAGACCCCAAGTGCTACATTTA
901 TAAGATTGGAATTGAAGCTGGTGTGTAATTAAGTAATAGACCCCAAGTGCTACATTTA    960
   TAACCTTTTGTAGCACCCCTGAACATAGTAATTTGTAGAGTTGGTGTGATTGTACATAA
961 TAACCTTTTGTAGCACCCCTGAACATAGTAATTTGTAGAGTTGGTGTGATTGTACATAA    1020
   TTGCAGTGGTTGGAACCTTGAAGACCCCTCCTCTGTATAATTTTAGTCTCTTCAGTGT
1021 TTGCAGTGGTTGGAACCTTGAAGACCCCTCCTCTGTATAATTTTAGTCTCTTCAGTGT    1080
   TCAGAGGAATGTGGTGATTAAAGGAGGAGTCTTAGTTTAAAGACTTTATATCAATTAATGA
1081 TCAGAGGAATGTGGTGATTAAAGGAGGAGTCTTAGTTTAAAGACTTTATATCAATTAATGA    1140
   TAATAAGGGAATGCTACTTTTACAAAAAATAAATAAATAAATAAATAAATAAATAAATAA
1141 TAATAAGGGAATGCTACTTTTACAAAAAATAAATAAATAAATAAATAAATAAATAAATAA    1184

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J. Gip16

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1   GAACCTCTCACACTACAACCTTCCCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT    60
   TTTCTGCTTTGCTAGTTCATGTCATGGCGTTTCTGAACCCCTTCTCTCTCCACATTCCCA
61  TTTCTGCTTTGCTAGTTCATGTCATGGCGTTTCTGAACCCCTTCTCTCTCCACATTCCCA    120
   M A F L N P F S L H I P M
121 TGTCCTTGTGCTCTTCCCTCTCTCTTTTTTTGCTTCTGCAAGCCAGTACTACTACTA    180
   S L L L F L L F F F C F C K A S T T T T
181 CCACCTCTACTACAACAAAGTCACTGTCCCTCTCTCTTCCCTCCACCTCACTACTCTCT    240
   T S T T T K S L S P S S S P S S T T T S S
241 CCTCTGATGTTCAACTTCTCTTGGGAAAGATCAAAGCTTCACTGCAAGGTAGTAACCTGT    300
   S D V Q L L L G K I K A S L Q G S N S D
301 ACAACCTTGTTTTGTCTCATGGAACCTCCACCCCACTTTGTGAGTGGAGTGGCCTCA    360
   N L V L S S W N S S T P L C Q W S G L K
361 AATGGGTCTTCTCCAATGGCACTCTCTCTCATGCACTGACTGTCTCTCCCAATGGA    420
   W V F S N G T P L S C T D L S S P Q W T
421 CCAATCTCACTCTCCACAAAGACCCTTCTCTTCACTTGTCTTCCCTTCGGCTCCCTCTG    480
   N L T L H K D P S L H L L S L R L P S A
481 CAAACCTCTGTGTTCCCTCCCAAGAGGCTTGGAGGTTCCCTATGCTCCAAAGTCTCT    540
   N L S G S L P R E L G G F P M L Q S L Y
541 ACCTTAACATAACTCATTTGGAGGTACCATCCCTCTTGAGCTTGGTTATAGCTCTCTCC    600
   L N I N S L E G T I P L E L G Y S S S L
601 TCTCTGAGATTGATTGGGTGACAAATAGCTAAGTGGGGTTCTTCCACCCCTCCATTTGGA    660
   S E I D L G D N M L S G V L P P S I W N
661 ACTTGTGTGAGAGGCTTGTTCCTTAGGCTCCATGGTAATTCCTATCTGAGGTGAGTT    720
   L C E R L V S L R L H G N S L S G S V S
721 CTGAGCTGCATTGCCTAACTCTTCTGCAAGAAATATCGAGTTCCTGATTGGGTGGCA    780
   E P A L P N S S C K N M Q L L D L G G N

```

Figure 3-2 continued

781	ACAAGTTCTCTGGAGTATCCCTGAGTTCATCACAAAGTTTGGTGGCCTAAAGCAGCTTG	840
	K F S G S F P E F I T K F G G L K Q L D	
841	ACTTGGGGAACAACATGTTTATGGGACAATTCCTCAAGGCCTAACTGGGCTTAGGCTTG	900
	L G N N M F M G T I P Q G L T G L R L E	
901	AAAAATGAATCTTTCACACAATACTTATAGCGGGTTTGGCCTTTGTTTGAGGAGAAT	960
	K L N L S H N N F S G V L P L F G G E S	
961	CCAAGTTTGGTGTGGATGCTTTTGGGGTAATAGCCCTAGCCTGTGTGGACCACTTTGG	1020
	K F G V D A F E G N S P S L C G P P L G	
1021	GAAGCTGTGCCAGGACCTCTACACTGAGTTCGTGCTGTGTGCTGGCATGTTATTAGTC	1080
	S C A R T S T L S S G A V A G I V I S L	
1081	TGATGACTGGTGCTGTAGTTTGGCTCTTTGCTGATAGGTATATGCAGAACAGAAGA	1140
	M T G A V V L A S L L I G Y M Q N K K R	
1141	GGGAGGGGAGTGGGAGAGTGAGGATGAGTTGAATGATGAAGAGGAAGATGATGAAGATA	1200
	E G S G E S E D E L N D E E E D D E D N	
1201	ATGGTGGCAATGCTATTGGTGGAGCTGGTGAGGGGAAGCTCATGTTGTTTGGTGGAGGTG	1260
	G G N A I G G A G E G K L M L F A G G E	
1261	AGAATTTGACATTGGATGATGTGTGAATGCAACTGGGCAGGTTTGGGAGCACTTGTGT	1320
	S L T L D D V L N A T G Q V L E K T C Y	
1321	ATGGGACAGCTTATAAGGCTAAGCTTGTCTGAAGGAGGCACCATTTGCTTTGAGGTTGTTGA	1380
	G T A Y K A K L A E G G T I A L R L L R	
1381	GAGAAGGTAGCTGCAAGACAAAGCTTCTTGTCTGTCTGTTATAAGGCAATGGGGAAAA	1440
	E G S C K D K A S C L S V I R Q L G K I	
1441	TTCCCATGAGAATTTGATTCTCTTTGAGAGCTTTCTATCAGGGGAAGAGAGGGGAGAAGC	1500
	R H E N L I P L R A F Y Q K G E K L	
1501	TCCTTATTATGACTACCTGCCTCTCAGAACCTTCATGATCTTTACATGAAGCTAAAG	1560
	L I Y D Y L P L R T L H D L L H E A K A	
1561	CTGGAAAACTGTGCTGAACCTGGGCTAGGCGACACAAGATTGCGTTGGGCGATGGCAAGAG	1620
	G K P V L N W A R R H K I A L G M A R G	
1621	GTCTAGCTTATCTTCATACAGGACTTGAAGTTCTCTGTCACTCATGCAAACGTAAGGTCCA	1680
	L A Y L H T G L E V P V T H A N V R S K	
1681	AGAATGTGCTCGTGGACGACTTCTTTCGCGCCAGGCTCACTGATTTGCGCCTGCACAAGC	1740
	N V L V D D F F A A R L T D F G L D K L	
1741	TGATGATTCCATCCATAGCCGACGAAATGGTGGCGCTTGCCAAGACGGATGGCTACAAGG	1800
	M I P S I A D E M V A L A K T D G Y K A	
1801	CTCCGAGCTTCAAAAGATGAAGAAATGCAACTCCAGGACTGACGTTTATGCGTTTCGGCA	1860
	P E L R M K K C N S R T D V Y A F G I	
1861	TACTGCTGCTGAAATCTTGATTGGGAAGAAGCCTGGGAAGAAGCAAGAAATGGGGAGT	1920
	L L L E I L I G K K P G K N G R N G E Y	
1921	ATGTGGACTTGCTTCAATGGTGAAAGTGGCAGTTTGGAGGAGACGACAATGGAAGTGT	1980
	V D L P S M V K V A V L E E T T M E V F	
1981	TTGATGTGCAACTTCTGAAAGGGATAAGAAGTCTTATGGAAGACGGGTTGGTGAGGCAC	2040
	D V E L L K G I R S P M E D G L V Q A L	
2041	TGAAGCTGGCAATGGGGTGTCTGTGCACCAAGTGGCATCTGTTAGGCCAAGCATGGATGAAG	2100
	K L A M G C C A P V A S V R P S M D E V	
2101	TTGTGAGGCAATTGGAGGAGAATAGACCAAGGAACAGGCTCTGCATTATACAGCCCTACAG	2160
	V R Q L E E N R P R N R S A L Y S P T E	
2161	AAACAAGAAGTGAAGTGTATACCCATTTTGAGCCCTTCAATTCAACAACATGCTTAAGC	2220
	T R S G S V T P F *	
2221	AAACTAGTGATTTTACTTACTACTTGTGTTTCTTTTCTCAGCTGAATCTGAATGTG	2280
2281	AGGGTAGCTATGATGGTCTAGAGAATGTTGTTATTTTGTGTTTACTCCATAACTCTTT	2340
2341	GCATTAGGTAGTCCCTATTGTTTTTGAATCAAAATATAGTCACACAATTATGCAAAATTC	2400
2401	TTGCTTCTGGAAAAAATAAAAAAAAAA	2428

Figure 3-2 continued

contains a complete open reading frame, and there is an in frame stop codon in the 5' untranslated region.

Both Gip6 and 14 (Fig. 3-2E and H), which encode ribosomal protein L37 and calmodulin-like protein, respectively, contain a complete open reading frame and have an in frame stop codon in the 5' untranslated region.

Gip3 (Fig. 3-2C) encodes serine acetyltransferase. Sequence comparison with genes in the GenBank (see Results in Chapter 4) suggested that it appears to have a complete open reading frame, though it does not contain an in frame stop codon in 5' untranslated region.

Gip12, 15, and 16 (Fig. 3-2G, I and J) encode cytochrome b561, translocase of outer mitochondrial membranes (TOM20), and transmembrane kinase-like protein (TMKL1), respectively (Heins and Schmitz, 1996, Srivastava et al., 1994, Valon et al., 1993). These proteins have been suggested to associate with the membrane. Cytochrome b561 has not to date been cloned from plants. Kyte-Doolittle analysis of GIP12 (sCyt b561) predicted six transmembrane segments that were similar in numbers and spacing to the known animal cytochrome b561 (data not shown) (Perin et al., 1988, Srivastava et al., 1994). Sequence analysis of soybean TMKL (sTMKL) encoded by Gip16 revealed that like its counterpart in *Arabidopsis thaliana* (Valon et al., 1993), it contains an amino terminal signal peptide, an extracellular domain with leucine-rich repeats, a single hydrophobic transmembrane segment, and a kinase-like intracellular domain which lacks, however, several of the conserved residues essential for catalytic activity (Hanks et al., 1988, Valon et al., 1993). sTMKL, however, differs from araTMKL1 in that it has a highly ser/thr-rich insert immediately following a signal peptide.

The Open Reading Frames of Seven Gips Are Out of Frame with the *LacZ* Gene

Uni-ZAP™ XR vector (Short et al., 1988) which is a derivative of Lambda ZAP® vector has a cDNA insert in a sense orientation (*EcoRI* - *XhoI*) with respect to the regulatable promoter of the *LacZ* gene which encodes β -galactosidase (β -gal). This positioning allows transcription of cDNA insert from *LacZ* promoter in the presence of IPTG, and production of recombinant fusion protein consisting of β -gal sequences linked to polypeptide sequences encoded by the cDNA insert. This permits the screening of the expression cDNA library constructed in Uni-ZAP™ XR vector.

Sequence analysis of Gip cDNAs revealed that three clones, Gip1, 5, and 15, were in the same reading frames as the *LacZ* coding sequence, but the other clones were not. Gip6 was in frame, but had a stop codon before the start of the putative open reading frame. Gip14 was in the reverse orientation. The other clones were in the proper orientation, but were not in the same reading frame as the *LacZ* coding sequence.

To investigate whether the reading frames of the latter seven clones were translated, cells transformed with pBluescript/Gips were induced with IPTG to express the recombinant proteins, and cell lysates were subjected to phosphorylation assays with GST-CDPK γ and resolved in SDS-PAGE. If recombinant proteins encoded by Gips were expressed and phosphorylated, phosphorylated bands with molecular weights similar to the predicted sizes (Table 3-1) should appear in the autoradiography (Fig. 3-3A). Proteins with molecular weights similar to those predicted for GIP1, 3, and 6 were phosphorylated by GST-CDPK γ (lane 1, 3, and 5, Fig. 3-3A). The expression of GIP1 was expected since the reading frame was in frame with the *LacZ* gene. The phosphorylation of proteins encoded by Gip3 and Gip6 suggested that the

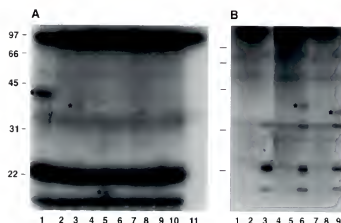


Figure 3-3. Phosphorylation of GIPs by GST-CDPK γ .

The cells transformed with pBluescript/Gips were induced with IPTG to express the encoded proteins. Cleared cell lysates were used in phosphorylation reaction with GST-CDPK γ , which was stopped by the addition of gel-loading buffer and subjected to electrophoresis in 12.5% SDS-gel. After staining with Coomassie Blue, the gel was dried and exposed to X-ray film. Coomassie staining showed each lane contained similar amount of total proteins (data not shown). In panel A, lane 1 GIP1; lane 2 GIP2; lane 3 GIP3; lane 4 GIP5; lane 5 GIP6; lane 6 GIP8; lane 7 GIP12; lane 8 GIP14; lane 9 GIP15; lane 10 GIP16; lane 11 GST-CDPK γ only. In panel B, cell lysates containing expressed GIP1 (lane 4 - 6) and GIP3 (lane 7 - 9) were phosphorylated with GST-CDPK α (lane 1, 4, and 7), - β (lane 2, 5, and 8), and - γ (3, 6, and 9) and treated as above. An extract of *E. coli* transformed with the empty pBluescript was used as a control (lane 1-3). Phosphorylated GIPs were marked with an asterisk.

reading frames for these clones, although not in frame with the *LacZ* gene, were utilized during translation in bacteria. Absence of phosphorylated bands with other clones, however, did not elucidate whether these clones were translated. Lack of phosphorylation could be ascribed to the unavailability of expressed proteins due to the low expression level or the formation of insoluble inclusion body, or possibly that the encoded proteins were interacting proteins rather than substrates, but it could not be excluded the possibility of no translation from these clones.

To further determine the specificity of phosphorylation, cell lysates containing recombinant GIP1, 3, and 6 were assayed with GST-CDPK α , β , and γ . Only GST-CDPK γ was able to phosphorylate GIP1 and 3 (Fig. 3-3B). This result was consistent with the data of interaction cloning. Neither of three CDPKs, however, phosphorylated GIP6 in this assay (data not shown). The reason why GIP6 was not phosphorylated was not determined.

Isolation of Additional 5' Sequence of Gip16

An additional 5' sequence of Gip16 was obtained by PCR cloning using primers to the Gip16 clone and a primer to the 5' side of cDNA insert on the UniZAP vector. The PCR template was the cDNA library employed in interaction cloning. The range chosen for the size fractionation of PCR products was based on a comparison between Gip16 and araTMKL1 sequence. With this method, a total of 462 bp were acquired. This sequence consisted of 284 bp as a new sequence and 178 bp as overlapping to the existing sequence.

GST-CDPK γ Interacts with and Phosphorylates Polyubiquitin but not Monoubiquitin

As shown in Fig. 3-3, GST-CDPK γ phosphorylated GIP1 which was a

polyubiquitin. To determine whether the phosphorylation was specific to the polyubiquitin, three different sources of monoubiquitins were tested in phosphorylation assay. Unlike the polyubiquitin, the recombinant (His)₆-monoubiquitin was not phosphorylated by GST-CDPK γ when cell lysates containing (His)₆-monoubiquitin were assayed in the kinase reaction. Neither bovine (Sigma) nor partially purified soybean ubiquitin were phosphorylated (data not shown). These results were confirmed in an experiment with GST-CDPK γ immobilized on glutathione-agarose (Fig. 3-4). Polyubiquitin, but not monoubiquitin, bound to the immobilized protein kinase.

Expression and Characterization of Recombinant GIPs

Five GIPs, 3, 6, 8, 14, and 16, whose identities were determined by the database search (Table 3-2) and whose activities were easily analyzable, were chosen for further study. GIP12 and 15 were not pursued in further study. These GIPs, except for GIP8, were expressed as fusion proteins linked either to maltose binding protein (MBP) or to (His)₆ at the amino terminus. The protein encoded by GIP8 was expressed as a native enzyme since it could be purified on glutathione-agarose column like other glutathione S-transferases (Simons and Vander Jagt, 1981). The purified recombinant GIPs were used for further characterization.

Phosphorylation

To confirm the results in Fig. 3-3 and to see whether other GIPs were able to be phosphorylated by GST-CDPK α , β , and γ , the phosphorylation assay was carried out with 2-6 μ g of purified recombinant GIPs. The results are summarized in Table 3-3. In the control assay, MBP alone was not phosphorylated by any of the CDPKs (data not shown). MBP-GIP3 (-sSAT),

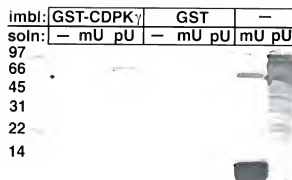


Figure 3-4. CDPK γ interacts specifically with polyubiquitin.

Ten micrograms of GST or GST-CDPK γ were immobilized on glutathione agarose. Cell lysates containing recombinant His₆-ubiquitin (mU) or a polyubiquitin (pU) were incubated with the agarose and after washing, bound proteins were eluted with glutathione. The eluted proteins were electrophoresed, transferred into nitrocellulose, and detected with antiubiquitin antibody. As a size control, untreated cell lysates containing either ubiquitin were immunostained (lanes with - in imbl).

-GIP6 (-sL37) were phosphorylated. This result agreed to the data that GIP3 and GIP6 were substrates of CDPKs. In contrast to earlier experiments, (His)₆-GIP14 (-sCaM) was phosphorylated. This implied that the cell lysates used in the previous experiment contained an amount of GIP14 that was below detection limit of the experiment. As shown in Fig. 3-3, GIP8 (sGST) and MBP-GIP16KD (-sTMKLKD), a deletion mutant that had an intracellular kinase domain but lacked both extracellular and transmembrane domain, were not phosphorylated by GST-CDPKs. It should be noted that unlike the results in Fig. 3-3, all three GST-CDPKs, α , β , and γ , phosphorylated MBP-GIP3. Possible reasons for this are discussed in Discussion.

Interaction

Since sGST and MBP-sTMKLKD were not phosphorylated, it was tested whether they interacted in solution with CDPKs. GST typically functions as either homo- or heterodimers (Marrs, 1996). Although heterodimerization

Table 3-3. Summary of phosphorylation of GIPs by CDPKs.

GIPs ^a	Phosphorylation ^b			GIPs	Phosphorylation ^b		
	α	β	γ		α	β	γ
GIP1	—	—	+	GIP8	—	—	—
GIP3	+	+	+	GIP14	+	+	+
GIP6	+	+	+	GIP16 ^c	—	—	—

^aFor GIP1 lysates of cells expressing pBluescript/Gip1 were used in phosphorylation assay. Other GIPs were expressed as fusion or native form, affinity-purified, and subjected to the assay.

^bPhosphorylation of GIPs was carried out with GST-CDPK α , β , and γ at room temperature for 15 min. The GIPs were electrophoresed in SDS-gel and autoradiographed.

^cGIP16 used in the assay was a deletion mutant that contained only intracellular kinase domain of GIP16.

between sGST and GST of GST-CDPKs seemed unlikely since sGST specifically interacted on the blot only with GST-CDPK γ during interaction cloning which employed GST-CDPK α , β , and γ , (His) $_6$ -CDPKs instead of GST-CDPKs were used in the experiment with sGST to avoid the possible complication on interpretation of the result. The interaction experiments were conducted with CDPKs as described in Experimental Procedure. The result showed neither sGST nor MBP-sTMKLKD interacted with CDPKs (data not shown). Since the interaction cloning was performed with autophosphorylated GST-CDPKs, the above experiment was repeated with autophosphorylated CDPKs. The results, however, were the same.

The experiments examining interaction in solution involved several washing steps and western blotting whose ranges of detection are tens to hundreds nanogram of antigens depending on the first antibodies. Thus, activity assays for CDPKs in the presence of sGST or MBP-sTMKLKD, which did not require washing steps or antibodies, were performed (data not shown). Neither sGST nor MBP-sTMKLKD at 1 to 1 molar ratio with (His) $_6$ -CDPK γ , affected the activities of the enzyme on phosphorylating its substrate, syntide-2. Inclusion of glutathione with sGST did not change the result. Since sGST was confirmed to possess glutathione S-transferase activity in assays with CDNB and glutathione as substrates (data not shown), it was tested whether the presence of autophosphorylated (His) $_6$ -CDPK γ affected the activity of sGST. The result showed sGST even at 1 to 50 molar ratio, was not affected by the presence of autophosphorylated (His) $_6$ -CDPK γ (data not shown).

Effects of phosphorylation

The effects of phosphorylation on the activities of MBP-sSAT, and -sL37 were examined. (His) $_6$ -sCaM which might be involved in regulation of

the activities of other proteins upon binding Ca^{2+} in analogy to the known functional roles of calmodulin, was not included in these experiments since possible proteins regulated by sCaM were not determined. Serine acetyltransferase (SAT) catalyzes the formation of *O*-acetyl serine from L-serine and acetyl CoA. It is feedback-inhibited by L-cysteine which is formed by cysteine synthase from *O*-acetyl serine (Kredich and Tomkins, 1966). Phosphorylation of MBP-sSAT by GST-CDPKs showed that phosphorylated MBP-sSAT was no longer sensitive to the inhibition by L-cysteine while the catalytic activity was not affected by the phosphorylation (data shown in Chapter 4). This effect was further studied in detail and described in Chapter 4.

Ribosomal protein L37, sL37, is a component of large subunit of the ribosome. The effect of the phosphorylation of sL37 on the efficiency of translation was analyzed using the wheat germ extracts in an *in vitro* translation assay (data not shown). The wheat germ extracts were pretreated with either GST-CDPKs and Ca^{2+} or Ca^{2+} alone as described in Experimental Procedures. The experiments, however, were hindered due to the presence of calcium-activated ribonucleases in the extracts. To avoid the inclusion of Ca^{2+} which is required for activation of GST-CDPKs, a constitutively active CDPK, GST-CDPK α (1-312) which is a deletion mutant that requires no calcium for activation, was used in the experiments. The result revealed that the translation was not influenced by the pretreatment of the extracts with GST-CDPK α (1-312). The wheat germ extracts contain endogenous CDPKs, but addition of staurosporin, an inhibitor of CDPK, to the translation assay had no effect (data not shown). Taken together, these results suggested that CDPK did not influence the translation of mRNA of small subunit light harvesting complex protein (ssLHCP) which was used in the experiments. However, it is premature to conclude that CDPK is not involved in the control of

translation, since it was not determined whether the endogenous L37 was phosphorylated by the CDPKs in the condition used in the experiment and since a specific mRNA was used in the experiment (see Discussion).

Characteristics of (His)₆-sCaM and MBP-sTMKLKD

As described in Table 3-2, sCaM has 40% identity to calmodulin, which is a calcium-binding protein that regulates the activities of other proteins upon binding to Ca²⁺. First, it was examined whether sCaM was able to bind Ca²⁺. The affinity-purified (His)₆-sCaM was resolved on a SDS-gel in the presence or absence of Ca²⁺ which was added to the sample buffer. Calmodulin typically shows a unique migration pattern due to the differences in the rates of migration on the SDS-gel, resulting from the differences in conformation in the presence and absence of Ca²⁺ (Grab et al., 1979, Klee et al., 1979). (His)₆-sCaM revealed Ca²⁺-dependent electrophoretic mobility shifts (data not shown). Second, since CDPKs possess a calmodulin-binding region, and binds calcium, it was examined whether sCaM affected the activities of CDPKs (data not shown). The results revealed that it did not affect either the catalytic activities of unphosphorylated or autophosphorylated CDPKs, or the autophosphorylation activities of CDPKs. Furthermore, inclusion of (His)₆-sCaM did not alter either K_{0.5} for Ca²⁺, the calcium concentration required for the half maximal activations of CDPKs or K_m for Syntide-2 (data not shown).

sTMKLKD was tested to see if it underwent autophosphorylation, which has often been used as an indicator that the protein kinase is active. sTMKLKD did not undergo autophosphorylation, which suggested it might not be an active enzyme. The inability was consistent with the prediction made from the sequence analysis above.

Discussion

CDPK is a large family of calcium-dependent protein kinases that are implicated to play important roles in the calcium signaling pathways in plants. To gain insights into the physiological roles of CDPKs, the present study attempted to identify substrates and interacting proteins of CDPKs, using interaction cloning with three different soybean CDPK isoforms labelled with ^{32}P by autophosphorylation (Harmon et al., 1987, Putnam-Evans et al., 1990).

This study describes isolation and characterization of twelve soybean cDNA clones, encoding proteins, GIPs, that specifically interacted with the GST-CDPK γ during interaction cloning. Sequencing revealed that all twelve clones encoded different proteins, which might result from the number of phage (150,000 pfu) screened and from the number (16 out of 35 clones) chosen for further study. Among ten clones (Gip4 and Gip13 were not pursued) eight were identified through the database search. A clone, Gip12 is the first cloning of cytochrome b561 from plants.

Sequence analysis showed that seven out of ten clones were out of reading frame with respect to the *Lac Z* coding sequence. Phosphorylation assays, however, revealed that at least two of them, Gip3 and Gip6, were translated in bacteria. A possible explanation is the fortuitous presence of a ribosome binding site in these clones. Or this might result from the mechanism that operates in bacterial operon

Phosphorylation of GIP1, 3 and 6 suggested that these were interacting proteins as well as possible substrates of CDPK γ . The non-phosphorylation of other GIPs did not exclude the possibility of their being substrates. When

GIP14 was overexpressed and subjected to phosphorylation, it was phosphorylated. This suggested non-phosphorylation was due in part to the experimental condition; unavailability because of inclusion body formation, low expression level, and resolution limit of SDS-gel; or they might be proteins that interact with CDPK *in vitro*.

Because GIP8 and 16 failed either to interact in solution or to affect the activities of CDPKs, it should first be established whether they are artifacts of the experiments or whether the inability of interaction and ineffectiveness on the activity of CDPKs were due to the experimental conditions. Using more sensitive methods, such as ECL (enhanced chemiluminescence) for the detection of interaction, stringent washing procedures and antibodies with high avidity would help to clarify the questions.

When overexpressed, affinity-purified GIP1, 3, 6, and 14 were used in phosphorylation assays, all of them were phosphorylated by all three CDPK isoforms. This result was in contrast to the earlier data. One possible explanation is the differences in K_m of these substrates for the three CDPKs. Since high concentrations of purified GIPs (2 - 10 μg) were used in later experiments, CDPKs, regardless of their K_m s, would phosphorylate the GIPs. In contrast, low concentrations of GIPs were present in extracts of bacteria expressing the clones originally isolated, and it is possible that only CDPK with low K_m s for the GIPs could phosphorylate them under these conditions. Another possibility is that there could be differences in the structure of GIPs in the two experiments since the GIPs used in later stages were fusion proteins. The conformation of MBP-fusion proteins might favor their phosphorylation.

There is no evidence in the literature that any of the proteins identified in this study (except calmodulin) are phosphorylated *in vivo*.

Phosphocalmodulin has been detected *in vivo* in animals, and tyrosine and ser/thr kinases phosphorylate calmodulin *in vitro*. Sacks et al. (1992) showed that phosphorylation of calmodulin *in vitro* by casein kinase II alters the activation of two calmodulin-dependent enzymes, namely myosin light chain kinase and cyclic nucleotide phosphodiesterase. Examination of possible effect of sCaM on the activities of CDPK indicated that unlike calmodulin, sCaM did not alter either the catalytic activities (V_{max} , K_m), or $K_{0.5}$ for calcium of CDPKs. Although it would be interesting if a separate calcium-binding protein (sCaM) acted as an allosteric regulator of CDPK, these negative results were consistent with the activation mechanism of CDPK described in Chapter 2, which suggested that calmodulin-like domain of CDPK is responsible for the regulation of CDPK in the presence of Ca^{2+} , and with the observation that addition of calmodulin does not change the activity of CDPK purified from soybean (Harmon et al., 1987). In order to test the effect of phospho-sCaM, it is necessary to identify the proteins that sCaM regulates.

Human ribosomal protein L37 is suggested in the literature to be phosphorylated (Barnard et al., 1994). The data, however, are solely based on the presence of phosphorylation motifs on the sequence. In contrast, several other ribosomal proteins, such as S6 (Stewart and Thomas, 1994) and acidic proteins (L45) (Naranda and Ballesta, 1991), are phosphorylated *in vivo* and *in vitro*, and the phosphorylation results in regulation of translation. The effect of phosphorylation of sL37 on translation was tested with sLHCP mRNA. The experiments, which showed pretreatment of wheat germ extracts with GST-CDPK α (1-312) ineffective on translation, suffered for technical reasons which resulted from the activation of ribonuclease by Ca^{2+} and thus, restricted the kind of CDPKs which could be assayed in the reactions. To examine properly the effect of phosphorylation of sL37 by CDPKs on

translation, it would be better to use reconstitution approach, which could also help to determine which CDPK is responsible for the regulation of translation. In addition, it is necessary to test whether the endogenous L37 is phosphorylated and to use total mRNA instead of using a particular mRNA, since there is a possibility that the phosphorylation controls translation of specific mRNAs (Stewart and Thomas, 1994).

Ubiquitin is a ubiquitous, well conserved, eukaryotic protein of 76 amino acids and acts as a tag for a ATP dependent protein degradation pathways (Gonda, 1994). The ubiquitin genes can be separated into two classes. The first codes for contiguous, spacerless, variable repeats of ubiquitin, and the second for ubiquitin with a carboxy-terminal extension of 52- 80 amino acids. Monoubiquitin is formed by the post-, or co-translational processing of multimeric or conjugated precursor proteins (Monia et al., 1990). Since CDPK interacts and phosphorylates only polyubiquitin, it will be interesting to test whether the phosphorylation affects the posttranslational processing. van Nocker and Vierstra (1993) observed that multiubiquitin chains linked through lysine 48 and glycine 76, which differs from polyubiquitin in which the linkage are between glycine 76 and methione 1, are present in plants, and are competent intermediates in the ubiquitin proteolytic pathway. It will also be interesting to test whether CDPK is able to recognize multiubiquitin and how phosphorylation affects ubiquitin-dependent proteolysis.

Cytochrome b561 is an electron transport protein. Its role has been well studied in animal cells where it is located in the membrane of neurosecretory vesicles and functions in supplying reducing equivalents to the intravesicular enzymes (Perin et al., 1988). In contrast, evidence of its presence in plants is largely lacking. A biochemical study showed that cytochrome b561 is present in the plasma membrane of plants (Asard et al., 1992). It was suggested that

cytochrome b561 may involve in electron transport in plasma membrane like it is in animal cells. There is evidence that CDPK controls the activity of membrane proteins, such as nodulin 26 or H⁺-ATPase (Roberts, 1993, Schaller and Sussman, 1988). It will be interesting to see how the interaction or phosphorylation affects the electron transport activity of cytochrome b561.

TOM20 is a surface receptor on the outer membrane of mitochondria, in which it exposes a large hydrophilic domain on amino terminus to the cytoplasm, and functions in the recognition of precursor proteins destined for import into mitochondria. This protein, thus, is involved in intracellular protein traffic (Moore et al., 1994). Pical et al. (1993) reported that the outer membrane of mitochondria contains more than 20 proteins which are phosphorylated in the presence of calcium. It was suggested that CDPK is involved in the phosphorylation of these proteins. Among these is a phosphoprotein which has a similar molecular weight to TOM20 (23 kDa). To establish whether sTOM20 is a substrate or an interacting protein of CDPK, it is necessary to show interaction and phosphorylation in vitro and in vivo. Eventually, analyzing the effect of interaction on mitochondria import will give an insight on the physiological significanes of the interaction.

TMKL is an unusual, putative transmembrane protein kinase (Valon et al., 1993). Its intracellular kinase-like domain was predicted to be non-functional since it lacks several key amino acids that are important for catalytic activity of protein kinases. The result that it did not undergo autophosphorylation is consistent with the prediction. The cDNA encoding this protein has been cloned from *Arabidopsis*, but there are no data indicating its possible role or location in plants (Valon et al., 1993). The present study showed its cDNA is present in soybean.

In summary twelve clones have been obtained by interaction cloning with CDPKs. Recombinant proteins encoded by four of these clones, Gip1, 3, 6, and 14 act as substrates of CDPKs. sSAT encoded by Gip3 showed that phosphorylation affects its allosteric regulation. Other clones, however, require additional studies to confirm whether they are artifacts of the experiments or they encode real interacting proteins. Furthermore, studies describing the interaction between GIPs and CDPKs in vitro and in vivo, requirement of autophosphorylation of CDPK on interaction, and specificities of CDPK isoforms, are required to fully address the possible physiological roles of the interaction.

CHAPTER 4 REGULATION OF SERINE ACETYLTRANSFERASE BY CDPK

Introduction

CDPK is a calcium-dependent, but calmodulin-independent protein kinase from plants. Its presence has been reported in a number of species ranging from plants to the protozoans. Although CDPK has been implicated as an important regulator in calcium-dependent signal transduction pathways, its physiological roles have not been well established. In this regard, it was attempted to isolate possible substrates and interacting proteins of CDPK by screening a cDNA library with ^{32}P -labelled CDPK in the previous study (Chapter 3). One of the interacting proteins identified was serine acetyltransferase which participates in cysteine biosynthesis.

Cysteine biosynthesis in plants represents, as it does in microorganism, the first step of incorporation of a reduced inorganic sulfur into L-cysteine, the first organic sulfur-containing compound (De Kok et al., 1993, Kredich and Tomkins, 1966, Schmidt and Jager, 1992). L-cysteine, then, serves as the principal starting metabolite for the synthesis of other sulfur-containing metabolites like methionine and glutathione. The biosynthesis of L-cysteine proceeds via a two-step enzymatic pathway (Kredich and Tomkins, 1966). Serine acetyltransferase (SAT) is responsible for the first reaction, the conversion of acetyl CoA and L-serine to O-acetyl-L-serine (OAS). The second step is catalyzed by cysteine synthase (CSase; also called OAS sulphydrylase,

OASS; or O-acetylserine(thiol)lyase), which converts OAS and sulfide to L-cysteine and acetate.

Both SAT and CSase have been purified, and cloned from microorganism (Kredich, 1993). The purification of CSase was reported from spinach (Droux et al., 1992) and carrot (Kuske et al., 1994). The molecular cloning of both enzymes, however, has lagged due in part to the low level of the enzymes, especially SAT, present in plants. A method termed "functional complementation" has made a major contribution in the isolation of genes of not only SAT and CSase, but also other enzymes in the sulfate assimilation pathway from plants (Leustek, 1996). This method allows the plant gene to be isolated based solely on the function of the enzyme it encodes, by adapting mutant microorganisms lacking specific sulfate assimilation enzymes.

Plant and bacterial SATs and CSases exist as a bifunctional complex, known as cysteine synthetase (Kredich, 1993, Ruffet et al., 1994, Saito et al., 1995). The complex can be disrupted in vitro by addition of OAS (Kredich and Tomkins, 1966). Reconstitution of the complex occurs spontaneously upon mixing solutions of each enzyme (Ruffet et al., 1994, Saito et al., 1995). The individual enzymes have catalytic activity and form homotropic complexes when isolated independently of the other enzyme. SAT is feedback inhibited by L-cysteine (Kredich and Tomkins, 1966, Saito et al., 1995) and this characteristic is thought to play a key role in the regulation of sulfate assimilation in bacteria (Kredich, 1993).

Intracellular localization studies in plants revealed that multiple isoforms of SAT and CSase are present in three different compartments, namely chloroplast, mitochondria, and cytosol (Kuske et al., 1996, Kuske et al., 1994, Ruffet et al., 1995, Takahashi and Saito, 1996). It was speculated that each isozyme participates in different metabolic functions in the plant cell and

responds differently to stimuli and demands for cellular metabolites. However, the physiological roles of each isoform in the different compartments are still unknown.

In physiological experiments, Rennenberg (1983) and Bogdanova et al. (1991) showed that the availability of OAS is rate-limiting for cysteine synthesis. Recently, by using transgenic tobacco overexpressing CSase, Saito et al. (1994) also supported the hypothesis that OAS is the important factor for regulation of cysteine biosynthesis. These observations strongly suggest that SAT, which produces OAS from serine and acetyl CoA, is a key enzyme in the final sulfur assimilation pathway.

In *E. coli*, a cysteine-excreting mutant was reported (Denk and Bock, 1987). This mutant has a mutation in the gene that encodes SAT which was found to render SAT less sensitive to feedback inhibition by L-cysteine. In plants there have been no reports of regulation of SAT activity other than by feedback inhibition. In this study, it was shown that soybean SAT (sSAT) was phosphorylated by CDPK, and its inhibition by L-cysteine was relieved by the phosphorylation. The regulatory roles of the phosphorylation are discussed in relation with the control of biosynthesis of cysteine and other sulfur-containing metabolites, especially glutathione.

Experimental Procedures

Materials

Acetyl coenzyme A (Acetyl CoA), L-serine, and L-cysteine were from Sigma. DU®-64 spectrophotometer, programming modules, and other accessories were purchased from Beckman. The *E.coli* strain, JM39, was

obtained from Dr. M. Berlyn (*E. coli* Genetics Stock Center, Yale University, New Haven).

Complementation Assay in *E. coli* JM 39

The *E. coli* strain used in the complementation assay was JM39 (F⁺, *cysE51*, *recA56*) (Jones-Mortimer, 1968), lacking serine acetyltransferase activity. This strain was maintained on either LB broth or M9 minimal medium (12.8 g Na₂HPO₄, 3 g KH₂PO₄, 1 g (NH₄)₂Cl₂, 0.5 g NaCl, 0.2 g MgSO₄•7H₂O, 1.1 mg CaCl₂•2H₂O per liter) supplemented with glucose (2 g/l) and L-cystine (100 µg/ml). The strain was tested for its Cys⁻ auxotrophic property before the complementation assay. The cells were transformed either with excised plasmid Clone3 (pBluescript/GIP3) or with pMal-cRI/GIP3. After 1 h incubation in LB media, the cells were washed twice in M9 medium and plated on M9 agar medium containing ampicillin (100 µg/ml) and IPTG (0.5 mM) at 37 °C.

Construction of Plasmids, Expression, and Purification of GIP3

Construction of expression plasmids and the expression conditions were described in Chapter 3. The plasmid, pMal-cRI/GIP3, encodes GIP3 as a fusion protein with maltose binding protein at the amino terminus. *E. coli* strains used for expression were JM39 or PR745. When JM39 was used as a host, a high molecular weight contaminant was copurified even after several chromatographic steps (data not shown). When PR745 was a host, however, this contaminant was absent after purification. Thus, PR745 was used as a host for subsequent expression experiments. Affinity purified MBP-GIP3 was further purified on Mono Q anion exchange column. The column was equilibrated with Buffer A (20 mM Tris, pH 8.0) supplemented with 0.15 M

NaCl. The protein was loaded onto the column and eluted with a gradient of 0.15 - 0.5 M NaCl in Buffer A. MBP-GIP3 was eluted at 0.3 - 0.4 M NaCl. The fractions containing MBP-GIP3 were combined and concentrated with Centricon 30 (Amicon) without a buffer change. For storage, glycerol and ethylene glycol were added to 40% and 10% (v/v), respectively and stored at -20 °C.

SAT Activity Assay

The SAT activity was determined by monitoring the decrease in A_{232} due to the thioester bond of acetyl-CoA (Leu and Cook, 1994b). The assay mixture contained 100 mM Tris, pH 7.5, 0.1 mM acetyl-CoA, 5 mM L-serine, and enzyme solution, in a final volume of 500 μ l. The reaction was initiated by the addition of L-serine. The temperature was maintained at 25 °C using a circulating water bath to heat the thermospacers of the cuvette compartment. The initial velocity of the decrease in absorbance at 232 nm was monitored. The L-cysteine inhibition assay was carried out as above except that 1 mM L-cysteine was included in the reaction mixture. Acetyl CoA was prepared at 50 mM in water and aliquots were stored at -80 °C. L-cysteine and L-serine were prepared fresh everyday at a concentration of 500 mM in 50 mM Hepes, pH 7.5.

Phosphorylation of MBP-GIP3 by GST-CDPK β

The phosphorylation reaction was carried out at 25 °C for 30 min in a 50 μ l mixture containing 50 mM Hepes, pH 7.5, 50 mM NaCl, 10 mM MgCl₂, 1 mM EGTA, 1.2 mM CaCl₂, 2 mM DTT, 60 μ M ATP, 0.1 mg/ml BSA, 6 μ g of MBP-GIP3, and 100 ng of GST-CDPK β . The reaction was stopped by adding 18 μ l of stop buffer (50 mM Hepes, pH 7.2, 90 mM EDTA, and 5 mM EGTA). The

total reaction mixture was desalted by gel filtration (10 DG, BioRad). The gel filtration column was equilibrated with 5 mM Hepes, pH 7.2. The phosphorylated MBP-GIP3 was used in SAT activity assay.

Determination of stoichiometry, IC₅₀ and K_m

For measurements of stoichiometry, the phosphorylation reaction in a volume of 25 μ l was performed as above except that [γ -³²P]ATP (500 cpm/pmol), 5 μ g of MBP-GIP3 and a total of 100 ng of GST-CDPK β were used, and an aliquot (10 μ l) was spotted on P81 paper at various time. GST-CDPK β was added at 5 time points during the course of the reaction at a concentration of 20 ng/ μ l. To determine the concentration of L-cysteine for 50% inhibition (IC₅₀) the phosphorylation reaction was carried out with 4 μ g of MBP-GIP3 and 5 ng of GST-CDPK β for 0.5 min in the presence of various concentrations of L-cysteine. GST-CDPK α , - β , and - γ were used in measuring K_m for MBP-GIP3 at a concentration of 25 ng, 10 ng, and 25 ng/25 μ l respectively. The reaction time was 1 min and various concentrations of MBP-GIP3 were used.

Sequence Analysis

Protein and DNA sequence analysis was aided by DNASTAR (Madison, WI) and by multiple sequence alignment program, CLUSTAL, provided by Genetics Computer Group (GCG) thru the Interdisciplinary Center for Biotechnology Research at the University of Florida.

Results

A cDNA Clone Identified by Interaction Cloning Encodes Serine Acetyltransferase

As described in Chapter 3, Gip 3 was identified as a CDPK γ -interacting protein by interaction cloning with purified recombinant GST-CDPK α , β , and γ labeled with ^{32}P by autophosphorylation. Sequence analysis of the entire 1.34 kb Gip3 cDNA insert revealed a putative open reading frame of 342 amino acids with a molecular mass of 37,046 Da.

Comparison of the deduced amino acid sequence of GIP3 to the GenBank database revealed homology to serine acetyltransferases (SAT) from plants (Bogdanova et al., 1995, Ruffet et al., 1995, Saito et al., 1995) and bacteria (Denk and Bock, 1987). The highest identity at the protein sequence level was 72% with *Arabidopsis* serine acetyltransferase (accession number, L34076). Serine acetyltransferases are involved in cysteine biosynthesis in plants and bacteria (Brunold, 1993, Kredich and Tomkins, 1966). Sequence comparison (Fig. 4-1) showed that the amino terminus has less homology whereas the carboxyl half has higher similarity. It was noted that acetyl-CoA binding site is present in this region of the enzyme (Ruffet et al., 1995, Saito et al., 1995). The protein encoded by the Gip3 was designated soybean SAT (sSAT).

GIP3 Functionally Complements Cys⁻ Auxotrophic *E. coli* Mutant

It was shown by the phosphorylation assay in Chapter 3 that Gip3 was translated in bacteria although it was out of frame with the *Lac Z* coding sequence. To further demonstrate that the longest reading frame was utilized to yield sSAT and to see that sSAT was functional, a functional

Figure 4-1. Alignment of amino acid residues of soybean serine acetyltransferase with known SAT.

Multisequence alignment was performed using a sequence analysis program, CLUSTAL, provided by Genetics Computer Group (GCG). Gaps introduced for optimal alignment are indicated by dashes. A star indicates absolutely conserved residue and a dot represents similar amino acids. The GenBank accession number for each sequence is L34076 for araSAT1, U22964 for araSAT2, D88529 for spinachSAT, and M15745 for *E. coli*SAT.

```

sSAT      1 -----MATCV
araSAT1   1 -----MATCI
araSAT2   1 MLPVTSRRHFTMSLYMLRSSSPHINHHSFLLPSFYSSKFHHHTLSPPSPPPPPMAACI
spInachSAT 1 -----MLAVSYAIPSCCI
E. coli SAT -----

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sSAT      6 DAPTPLSRKPNQSSQSS---DDRSFNMYK-----FCRPTGSDRVPICPICNKRDTAFT
araSAT1   6 DTCRTGNTQD-----DDSRFCCIKN-----FFRPGFS-----VN
araSAT2   61 DTCRTGKPIISPRDSSKHHDDESGFRYMNY-----FRYPDRSS-----FNGTQT
spInachSAT 14 NRFYIREFSSSLOPALHHPWYKKRFCCSTNOENCSLGSPTHLRPM-----GGDLS
E. coli SAT 1 -----MSCEEL-----

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sSAT      54 RVEEFDTCVEDNDDIEVEVVEGVDLWLKIOEEARLDVDOEPILSSYYFSSILSHKSLESA
araSAT1   35 RK1IHHTQIEDD-----DDVWIKMLEEAKSDVKQEPILSNYYIASITSHRSLSEA
araSAT2   105 KTLHTRPLLEDLD---RDAEVDVWAKIRREAKSDIAKEPIVSAYYHASIVSORSLEAA
spInachSAT 66 VAPSYGHLTANN-----EAWLWDQIKGEARRDADSEPALASYLSTILSHSLSERS
E. coli SAT 7 -----ETVWNNIKAEARTLADCEPHLASFYHATLLKHENLGS

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sSAT      114 LANHLSTNLSSLSPSTLSELFHGVLDVDDGDDIVGAVKDDL IAYKERDPACISYVHCL
araSAT1   84 LAHILSVKLSNINLPSNTLSELFISVLE--ESPEI IESTKODL IAYKERDPACISYVHCF
araSAT2   161 LANTLSVKLSNINLPSNTLFDLFSGLVQ--GNPDI VESVKLDL IAYKERDPACISYVHCF
spInachSAT 117 LSFHLGNKLCSTLLSTLLYDLFLNLS--SDSSLLDAVADLRAARVRDPACVSFSHCL
E. coli SAT 45 LSYMLANKLSSPIMPAIATIREVVEEAYA--ADPEH IASAACDIQAVRTDPAVDKYSTPL

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sSAT      174 LNFKGFLACQAHRIAHLWLQGRKVLALLIONRVSEFAVDIHPGAKIGRGILLDHATGL
araSAT1   142 LGFGKFLACQAHRIAHTLWKNRKIVALLIONRVSEFAVDIHPGAKIGKGILLDHATGV
araSAT2   219 LHFKGFLACQAHRIAHELWTDORLILALLIONRVSEFAVDIHPGAKIGTGILLDHATAI
spInachSAT 175 LNYKGFLACQSHRVAHKLWNODRRPLALALHSRISDVFAYDIHPAARIGKGILLFDHATGV
E. coli SAT 103 LYLKGFHALQAYRIGHWLWNOGRRALAI FLQNOQSVTFQVDIHPAAKIGRGIMLDHATGI

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sSAT      234 VYGETAVIGNNVSILHNVTLGGTGKASGDRHPKIGDGVLIAGATC ILGNIKIGDGAKIGA
araSAT1   202 VIGETAVVGNVSILHGVTLGGTGKQSGDRHPKIGDGVLIAGASC ILGNITIGEGAKIGS
araSAT2   279 VIGETAVVGNNVSILHNVTLGGTGKQSGDRHPKIGDGVLIAGATC ILGNITIGEGAKIGA
spInachSAT 235 VIGETAVIGDNCSILHHVTLGGTGKAGGDRHPKVGDGVLIAGATC ILGNVRIIGDGAKIGA
E. coli SAT 165 VYGETAVIENDVSILOSVTLGGTGKSGDRHPKIREGVHIGAGAK ILGNIEVGRGAKIGA

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sSAT      294 CSVVLKEVPPRTTAVGNPARLVGGKDNPIKLDKMPSTMDHTS---WSDYVI
araSAT1   262 GSVVVKDVPARTTAVGNPARLIGGKENPRKHOKIPCLTMDOTSYL TEWSDYVI
araSAT2   339 GSVVLKDVPRTTAVGNPARLIGGKDNPKTHOKIPCLTMDOTSHISEWSDYVI
spInachSAT 295 GSVVLIDVPPRTTAVGNPARLIGGKEKPSQNSDVPGESEMDHTSFI SEWSDYVI
E. coli SAT 223 GSVVLQVPVPHHTAAGVPAIRIVG-KPDSDKPSMDMDQHFNGINHTFEYGDGI-

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complementation assay was performed. This approach uses mutant *E. coli* lacking specific enzymes to isolate the plant gene based solely on function of the enzyme it encodes, and it has been successfully employed to identify serine acetyltransferases from plants (Bogdanova et al., 1995, Murillo et al., 1995, Ruffet et al., 1995, Saito et al., 1995). The *E. coli* mutant, JM39, which lacks SAT, was transformed with pBluescript/Gip3 and plated on minimal media lacking L-cysteine and supplemented with 100 mg/ml of ampicillin. All colonies that carried pBluescript/Gip3 were able to grow in the absence of cysteine, whereas controls with JM39 that were not transformed or transformed with pBluescript alone were unable to grow without addition of cysteine (data not shown). This result verified that the longest reading frame was translated and the product had serine acetyltransferase activity.

Phosphorylation Makes sSAT Insensitive to Allosteric Inhibitor, L-cysteine

When pBluescript/Gip was induced to express recombinant sSAT, the level of expression was very low. To overcome a low expression level and to facilitate the purification of recombinant protein, sSAT was expressed under the control of the strong promoter and as a fusion protein with maltose binding protein at the amino terminus. The purified recombinant MBP-sSAT was used in phosphorylation assays to demonstrate sSAT is a substrate of CDPKs. Surprisingly, all three CDPKs were able to phosphorylate MBP-sSAT (see below). The control experiment with MBP as a substrate showed that MBP was not a site for phosphorylation (data not shown). The highest phosphorylation stoichiometry attained with GST-CDPK β was 0.97 mol of phosphate/mol of MBP-sSAT. This suggested that there might be one potential phosphorylation site present in sSAT (see below).

The effect of phosphorylation was investigated using the phosphorylated MBP-sSAT. To remove ATP which interfered the spectrophotometric assay of SAT, the phosphorylated MBP-sSAT was desalted prior to SAT activity assay. The activity of MBP-sSAT was not affected by the phosphorylation (Fig. 4-2). Phosphorylation, however, did affect the sensitivity of MBP-sSAT to L-cysteine. L-cysteine, a final product of the biosynthetic pathway, acts as an allosteric inhibitor of SAT in a noncompetitive manner (Kredich and Tomkins, 1966, Saito et al., 1995). Untreated MBP-sSAT was almost completely inhibited by 1 mM L-cysteine, whereas phosphorylated MBP-sSAT was insensitive to the presence of L-cysteine. This effect was similar no matter which GST-CDPK was used in the phosphorylation reaction.

L-cysteine Protects SAT from Phosphorylation by CDPK

Interestingly, it was observed that L-cysteine exerted an inhibitory effect on the phosphorylation of sSAT by CDPK. The concentration for 50% inhibition (IC_{50}) was 3 μ M (Fig. 4-3). This effect was observed only when MBP-sSAT was the substrate. Phosphorylation of syntide-2 was not inhibited by L-cysteine even at a concentration of 1 mM (data not shown). This result indicated that the inhibitory effect of L-cysteine on CDPKs was through its interaction with MBP-sSAT rather than with CDPKs.

sSAT Contains One Possible Phosphorylation Site at the Carboxyl Terminus

CDPKs have preferred phosphorylation motif, basic - X - X - Ser/Thr where basic indicates basic amino acids and X for any amino acids (Roberts and Harmon, 1992). The motif for phosphorylation was searched on sSAT. There was only one site that satisfied the requisite of the phosphorylated

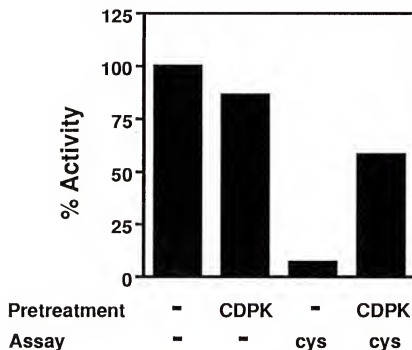


Figure 4-2. Phosphorylation makes sSAT insensitive to the feedback inhibition by L-cysteine.

MBP-sSAT was pretreated with or without GST-CDPK β in the presence of ATP for 15 min at room temperature. The reaction mixtures were desalted and used in SAT assay, which was carried out in the presence or absence of 1 mM L-cysteine.

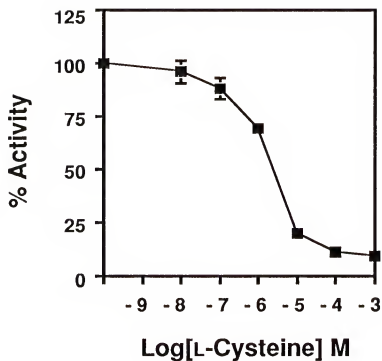


Figure 4-3. Determination of IC_{50} for GST-CDPK β with L-cysteine.

The concentration for 50% inhibition was determined with MBP-sSAT (1.26 μ M) as a substrate in the presence of various concentration of L-cysteine.

motif (Fig. 4-4). This agreed with the study of phosphorylation stoichiometry (see above). Lee and Harmon (unpublished data) noted that CDPKs prefer hydrophobic, branched-chain amino acids at positions P-5, P+1, and P+4. The proposed motif in sSAT has a leucine residue at P-5, but the other two positions have different amino acids.

Determination of K_m

During interaction cloning, sSAT interacted only with GST-CDPK γ and in the initial phosphorylation experiment sSAT was phosphorylated only by GST-CDPK γ . However, when purified recombinant MBP-sSAT was used in the phosphorylation assay, all three CDPKs, GST-CDPK α , β , and γ , were able to phosphorylate MBP-sSAT. One possibility was three enzymes might have different K_m s for MBP-sSAT. This possibility was appealing since the concentration (2-6 μ g) of purified recombinant MBP-sSAT used in the later experiment was much higher than the initial experiments. Determination of $K_{m,app}$ was attempted for all three CDPKs (data not shown). Double reciprocal plots, however, showed curvature for all three CDPKs and could not be used to calculate the $K_{m,app}$.

Discussion

This study describes the isolation of a cDNA clone encoding a soybean serine acetyltransferase (sSAT) by interaction cloning, identification of sSAT as an in vitro substrate of CDPK, and the characterization of sSAT in relation with the effect of phosphorylation.

CDPKs, α , β , and γ , were able to phosphorylate MBP-sSAT and rendered phospho-MBP-sSAT to be insensitive to the feedback inhibition by L-cysteine. The phosphorylation stoichiometry with GST-CDPK β was 0.96 mole of

Syntide-2	PLARTLSVAGLPGKK
sSAT (321-334)	PIKLDKMPSFTMDH
	-5 +1 +4

Figure 4-4. sSAT has one possible phosphorylation site for CDPK.

A phosphorylation motif (Basic, X, X, S/T, where Basic represents basic amino acids, X for any amino acids, S for serine, and T for threonine) in sSAT was compared with Syntide-2. Residues are numbered relative to the phosphorylated residue at position P. Residues amino terminal to P have negative numbers and those carboxyl terminal to P have positive numbers

phosphate/mole of MBP-sSAT, which suggests SAT may have one phosphorylation site. This hypothesis is further supported by the finding that there is only one phosphorylation motif for CDPK (Roberts and Harmon, 1992). Interestingly, when sSAT and *E. coli* SAT was aligned, the serine (amino acid residue, 329) in this motif was aligned in proximity to a methionine (256) of *E. coli* SAT, whose mutation resulted in a less-sensitive *E. coli* SAT to feedback inhibition (Denk and Bock, 1987). Taken together, the data imply that this region is involved in feedback inhibition. Sequence comparison with known plant SATs showed that the phosphorylation motif is present only in the sSAT. This, however, does not imply that the regulation by phosphorylation is unique to sSAT since plant SATs are encoded by multigene families (Roberts and Wray, 1996, Ruffet et al., 1995) and thus, regulated SAT may have not yet been isolated in other species.

Surprisingly, L-cysteine inhibited phosphorylation of sSAT by GST-CDPK β . This effect, however, was restricted to SAT and not to another substrate of CDPK, which indicated L-cysteine interacted with SAT and interfered the phosphorylation. This inhibitory effect is intriguing, considering the cellular concentration, 10 μ M, of L-cysteine (Giovanelli, 1990). At this concentration CDPK is unable to phosphorylate sSAT since its IC₅₀ for L-cysteine is 3 μ M. This implies that unless there are circumstances (see below) that require rapid consumption of L-cysteine which lower the intracellular concentration of L-cysteine and that increase intracellular Ca²⁺, cells do not permit CDPK to phosphorylate sSAT.

In bacteria (Kredich, 1993) and plants (Neuenschwander et al., 1991, Rennenberg, 1983, Saito et al., 1994), it has been noted that the concentration of L-cysteine is controlled very tightly, and SAT and its product, OAS, are important factors for controlling L-cysteine biosynthesis. Bacteria regulate L-

cysteine synthesis in two ways: 1) feedback inhibition of SAT activity by L-cysteine; 2) activation of cysteine regulon by OAS (Kredich, 1993, Kredich and Tomkins, 1966). Recently, plant SAT was reported to be feedback-regulated by L-cysteine (Saito et al., 1995). The effect of OAS on the regulation of gene expression has not been reported from plants. The present study suggests that there is yet another type of regulation on L-cysteine biosynthesis in plants.

L-cysteine is required as precursors not only for the primary plant metabolites, but also for the synthesis of glutathione (GSH), one of the most abundant sulfur-containing compounds in plants. Glutathione is a major cellular reducing agent and protects the cell from oxidative damage caused by a number of environmental stress, such as pathogen attack, wounding, cold, and heavy metals (Hausladen and Alscher, 1993). It has been shown that increase in active oxygen species such as H_2O_2 in the cell stimulates GSH synthesis (Smith, 1985, Smith et al., 1984, Smith et al., 1985). Using aminotriazole which inhibits catalase, May and Leaver (1993) demonstrated that mild oxidative stress can stimulate GSH level.

H_2O_2 has been demonstrated to stimulate a transient increase in Ca^{2+} concentration. Soybean cells, when treated with H_2O_2 , display a rapid influx of Ca^{2+} (Levine et al., 1996). With recombinant aequorin techniques, Price et al. (1994) reported that H_2O_2 increases intracellular Ca^{2+} in tobacco seedlings. Recently McAinsh et al. (1996) showed that the oxidative stress increases Ca^{2+} in guard cells, which results in inhibition of stomatal opening and promotion of stomatal closure and this effect is reversed by EGTA.

The current working hypothesis for the physiological implication of phosphorylation of sSAT by CDPK is that the oxidative stress increases intracellular free calcium, which activates CDPK whose phosphorylation on sSAT results in deregulation of feedback inhibition and supplying L-cysteine

for the synthesis of GSH. Further studies describing the phosphorylation of sSAT in vivo upon the oxidative stress and the connection of CDPK to the responsible phosphorylation are necessary to test the hypothesis. In addition, study on the specificity of CDPK isoforms for phosphorylating sSAT in vitro and in vivo will give an insight into establishing the responsible CDPK isoform in the regulation of L-cysteine biosynthesis.

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BIOGRAPHICAL SKETCH

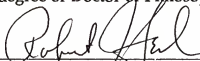
Byung-Chun Yoo was born May 6, 1964, in Kyunggi Prov., Korea and grew up in Seoul, Korea. He attended Korea University in Seoul, Korea, receiving a Bachelor of Agriculture in February of 1989. After spending time in the Genetic Engineering Center in Seoul as a lab technician, Byung-Chun was admitted to the University of Florida in the fall of 1990. He began graduate school in the Department of Botany under the supervision of Dr. Alice C. Harmon. He was earned a Master of Science degree in August, 1992. Then, he continued his graduate work for the Ph.D. degree in the laboratory of Dr. Alice C. Harmon but changed the major to the Graduate Program in Plant Molecular and Cellular Biology. He is going to complete the requirements for the degree of Doctor of Philosophy in May, 1997. He will work for Dr Alice C. Harmon until August, 1997 and join Dr. William Lucas in the Plant Biology Section at the University of California at Davis as a postdoctoral fellow.

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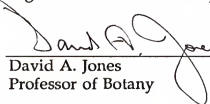
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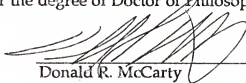
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May, 1997

Dean, Graduate School